University of Alberta

The role of matrix metalloproteinases in inflammatory cardiovascular diseases

by

Manoj Mathew Lalu ©

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of the requirements for the degree of Doctor of Philosophy

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Abstract

An imbalance between matrix metalloproteinases (MMPs) and their endogenous inhibitors (tissue inhibitors of MMPs, TIMPs) is implicated in many long-term cardiovascular diseases, however their involvement in short term inflammatory cardiovascular dysfunction remains unknown. Since MMPs are regulated by proinflammatory cytokines and oxidative stress, I investigated whether MMP activity is affected in two models of cardiovascular inflammation: 1) a rat model of endotoxemia, and 2) ischemia-reperfusion (I/R) injury in isolated rat hearts and patients undergoing cardiac surgery.

In endotoxemic rats, hypotension occurred in the first 24 h following lipopolysaccharide (LPS) injection. When blood pressure was lowest (6-12 h) MMP-2 activity was depressed in plasma and heart tissue, while MMP-9 activity was increased in the plasma. Circulating MMP-9 activity correlated negatively with blood pressure at all time points following LPS administration.

The effect of MMP inhibition in endotoxemic cardiac mechanical dysfunction was investigated. Cardiac mechanical function, measured *ex vivo*, was depressed 6 h after LPS administration. This was associated with decreased ventricular and perfusate MMP-2, and increased perfusate MMP-9 activities. Administration of MMP inhibitors following LPS injection decreased perfusate MMP-9 activity and ameliorated the cardiac dysfunction.

The involvement of MMPs in inflammatory vascular contractile dysfunction was also investigated. In isolated rat aorta, MMP inhibitors prevented LPS and interleukin-1β

induced vascular contractile dysfunction. In endotoxemic rats net aortic gelatinolytic activity was increased 6 h after LPS administration. MMP-2 and MMP-9 activities were increased, while TIMP-1 protein content was enhanced and TIMP-4 diminished.

Our group has demonstrated that activation of MMP-2 is involved in myocardial I/R injury. I found that ischemic preconditioning, which ameliorates I/R injury, decreased MMP-2 activation and release from isolated rat hearts. In patients undergoing coronary artery bypass grafting with cardiopulmonary bypass, immediately following reperfusion MMP-2 and MMP-9 activities increased in the myocardium and plasma, while myocardial TIMP-1 decreased. Myocardial MMP activities positively correlated with the time of ischemia and also the severity of resulting cardiac dysfunction.

In summary, I have found that MMP activities are regulated by endotoxin and I/R induced injuries. Inhibition of MMPs may prove to be a novel therapeutic approach to treat acute inflammatory disorders.

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List of Abbreviations

° C	degrees Celsius
>	greater than
<	less than
=	equal
ANOVA	analysis of variance
BCA	bicinchoninic acid
eNOS	endothelial nitric oxide synthase
ET	endothelin
GSH	glutathione
h	hour
HPLC	high performance liquid chromatography
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
I/R	ischemia-reperfusion
K_{ATP}	ATP gated posstium channel
kDa	kilodalton
kg	kilogram
LPS	lipopolysaccharide
mg	milligram
µg	microgram

mL	milliliters
mmHg	millimeters mercury
min	minute
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MT-MMP	memebrane type matrix metalloproteinase
NAD(P)H	nicotine adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
NO	nitric oxide
NO_x^-	nitrate/nitrite
$ONOO^-$	peroxynitrite
PE	phenylephrine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of mean
TIMP	tissue inhibitor of matrix metalloproteinase
TNF	tumor necrosis factor
TnI	troponin I
v/v	volume by volume
w/v	weight by volume

CHAPTER 1

INTRODUCTION

Portions of the chapter are in press. Lalu MM, Leon H, Schulz R. Turmoil in the cardiac myocyte: acute intracellular activation of matrix metalloproteinases. In: Villarreal F, ed. *Interstitial Fibrosis*. San Diego: Kluwer Academic Publishers; 2003.

In this thesis, I have explored whether matrix metallproteinases (MMPs), a family of endopeptidases, are involved in acute inflammatory cardiovascular disease. Although MMPs have been implicated in a number of chronic pathologies (e.g. cancer, atherosclerosis, heart failure) it remains to be resolved if they have actions which are of importance in acute conditions. I have investigated the potential involvement of MMPs in two such conditions, endotoxemia and myocardial ischemia-reperfusion (I/R) injury.

In this introductory chapter I will review the topics of MMPs, endotoxemia, and myocardial I/R injury. Section 1 will provide a general introduction of MMPs and detail the evidence which implicates them in long-term cardiovascular pathologies. Section 2 will give an overview of endotoxemia (and its closest clinical correlate, septic shock), and review the evidence which supports a role for MMPs in this disorder. The next section will provide an overview of the evidence linking MMPs to I/R injury in animal models. Finally, in the last section I will outline the questions and hypotheses which underlie each of the subsequent chapters.

1.1 Matrix metalloproteinases

1.1.1 History and nomenclature

In 1962 Jerome Gross and colleagues first described an "activity" which was present during the maturation of tadpoles and had the ability to degrade rods of collagen into ¾:¼ length fragments.[1] In retrospect, this activity is now recognized to be that of interstitial collagenase, one member of a family of enzymes called 'matrix metalloproteinases' (MMPs). Traditionally, MMPs are believed to degrade components of the extracellular matrix in a concerted manner, allowing for such diverse processes as embryogenesis, atherosclerosis, and wound healing.

MMPs are a large family of enzymes, with over twenty mammalian members now described in a variety cell types. They are classified by numerical designation (MMP-1 through MMP-28) and also according to their *in vitro* substrate specificity. Thus far, a number of different classes have been described: collagenases (MMPs -1, -8, and -13), stromelysins (e.g. MMP-3), membrane-type MMPs (MT-MMPs, 1 through 8), gelatinases (MMP-2 and -9), and of other MMPs (e.g. matrilysin). Many of these MMPs have been identified in a variety of cardiovascular cell types as detailed in Table 1.1.

Table 1.1 Synthesis of MMPs and TIMPs in cells relevant to the cardiovascular system

Cell Type	MMP	Reference
Cardiac myocytes	MMP-2	2
	MMP-3	3
	MMP-9	3
	TIMP-1	4
	TIMP-2	4
	TIMP-3	4
	TIMP-4	4,5
Cardiac fibroblasts	MMP-1	6,7
	MMP-2	6,8-11
	MMP-3	8
	MMP-9	9,10
	MMP-13	9
	MT-1 MMP (MMP-14)	8
	TIMP-4	12,13
Endocardial cells	MMP-2	14
	TIMP-2	15
	TIMP-3	15
Smooth muscle cells	MMP-1	16,17
	MMP-2	18-21
	MMP-3	17
	MMP-9	16-19,21
	MMP-12	22
	MT-1 MMP (MMP-14)	23
	MT3-MMP (MMP-16)	23,24
	TIMP-1	21,25
	TIMP-2	21
	TIMP-3	21
Endothelial cells	MMP-1	26-28
	MMP-2	10,26,28,29
	MMP-3	26
	MMP-9	10,29
	MT-1 MMP (MMP-14)	27,28,30
	TIMP-1	26,31
	TIMP-2	26,31
Platelets	MMP-1	32
	MMP-2	33-35
	MMP-9	35
	MT-1 MMP (MMP-14)	34
	TIMP-1	34
	TIMP-2	34
	TIMP-4	36

1.1.2 Structure and activation

Regardless of *in vitro* substrate preference, all the MMPs are initially synthesized in a zymogen form and have quite similar domain structures.[37] Starting at the N-terminus, most MMPs have a signal peptide which allows for secretion into the endoplasmic reticulum and eventual transport out of the cell. Next to the signal peptide, most MMPs have a hydrophobic propeptide domain which shields the catalytic domain next to it. Finally, at the C-terminus most MMPs also have a hemopexin domain which confers some substrate specificity and allows docking with other proteins. The catalytic domain of all MMPs is known as the 'matrixin fold' and consists of five-stranded beta-sheets and three alpha-helices. This fold forms substrate binding pockets, coordinates with the catalytic Zn^{2+} ion, and also binds two Ca^{2+} ions.[38] In its zymogen form, the catalytic Zn^{2+} is coordinated to a cysteinyl sulphydryl group on the propeptide domain (a so called 'cysteine switch'[39]) and is rendered inactive.

Since MMPs are initially synthesized with the propeptide domain shielding the matrixin fold, they must be activated to expose the catalytic Zn^{2+} ion. To date, four different mechanisms have been described: a) stepwise activation in the extracellular space, b) activation at the cell surface by MT-MMPs, c) intracellular activation, and d) activation by oxidative stress. Although most of these mechanisms have not been specifically elucidated in any type of cardiac cells, it is generally believed that MMP activation occurs by similar means regardless of the cell type.

In the extracellular stepwise activation process, another proteinase (such as plasmin, trypsin, elastase, or an MMP) cleaves at a susceptible loop region (which acts as 'bait') in the propeptide domain of the MMP. Upon cleavage, the prodomain structure breaks down and its shielding of the catalytic cleft is withdrawn. Water is then allowed to enter and hydrolyze the coordination of the cysteine to the Zn^{2+} ion. Ultimately, this renders the MMP prone to autocatalytic activity which cleaves off the propeptide domain and produces a lower molecular weight active enzyme.[38,40]

Alternatively, MMPs may also be activated at the cell surface by MT-MMPs. In order for MT-MMP1 to process MMP-2, MT1-MMP forms a complex with tissue inhibitor of matrix metallproteinase-2 (TIMP-2), which serves as a receptor for MMP-2. When MMP-2 docks with this complex, proteolytic activation occurs at the cell surface, and an active MMP is released from the cell. [41] This method of activation would allow for increased MMP activity within the immediate pericellular space and, as noted in MT1-MMP knockout mice, is important for MMP-2 activation.[42] Specifically, fibroblasts derived from MT1-MMP-knockout mice were unable to show MMP-2 activity in response to stimulation with collagen.[43]

In contrast to extracellular or pericellular activation, a number of MMPs (e.g. MMP-11, MT-MMPs) are activated intracellularly by furin-like proprotein convertases.[44-46] After intracellular activation has occurred, the active MMP is shuttled either to the cell membrane for insertion (in the case of MT-MMPs) or secretion (e.g. MMP-11). Thus, in these circumstances, intracellular activation does not necessarily lead to intracellular activity of MMPs.

A final pathway of MMP activation, which can occur both extracellularly and intracellularly, is oxidative stress (Figure 1.1). In this latter mechanism, a variety of endogenous oxidants (e.g. superoxide anion, hydrogen peroxide, peroxynitrite) oxidize the sulphydryl bond of the cysteinyl group which binds to the catalytic Zn^{2+}. As with other mechanisms, disruption of this inhibitory bond allows hydration of the catalytic site. However, unlike other mechanisms, oxidative activation produces an 'activated proenzyme' in which the propeptide domain has not been removed.[47,48]

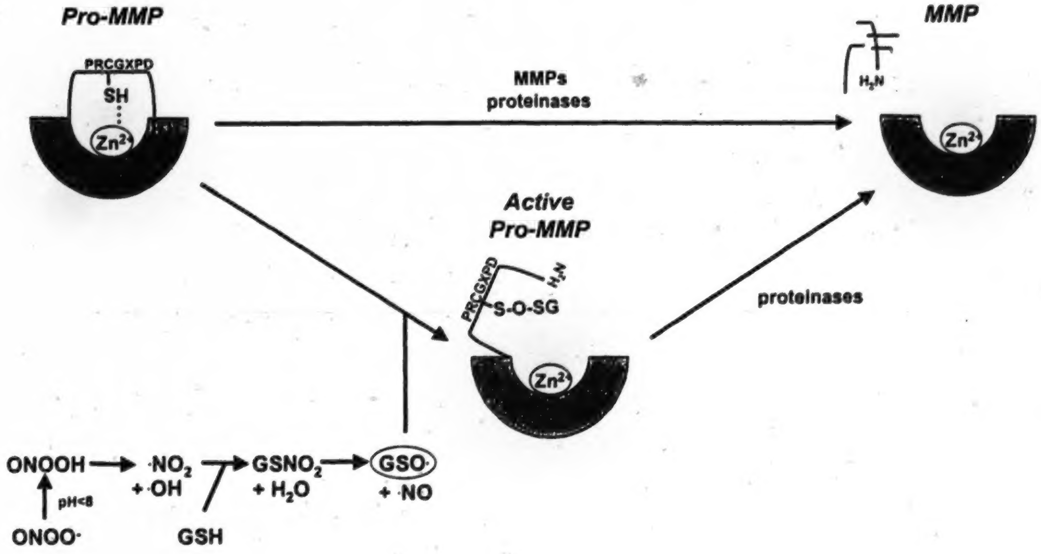

Figure 1.1 Activation of MMPs by oxidative stress. ProMMPs are synthesized with a propeptide domain shielding the catalytic Zn^{2+}. ProMMPs can be activated by classic proteolytic cleavage of its propeptide domain by MMPs or other proteinases (top arrow). Alternatively, MMPs can be activated by oxidants like peroxynitrite which interfere with the propeptide domain's ability to coordinate with the catalytic Zn^{2+} ion. Peroxynitrite ($ONOO^-$), in an aqueous environment, forms the species ONOOH which spontaneously releases nitrogen dioxide radical ($^{\cdot}NO_2$) and hydroxyl radical ($^{\cdot}OH$). Glutathione (GSH, a tripeptide sulfhydryl containing compound) reacts with nitrogen dioxide radical and hydroxyl radical to form S-nitroglutathione ($GSNO_2$) and water. A concerted rearrangement of the S-nitroglutathione then occurs [$GSNO_2 \rightarrow (GS^{\cdot}, {}^{\cdot}NO_2) \rightarrow GSONO \rightarrow GSO^{\cdot} + NO^{\cdot}$] and GSH sulfinyl radical ($GSO^{\cdot}$) is produced along with nitric oxide ($NO^{\cdot}$). GSH sulfinyl radical can S-glutathiolate the cysteine (thiol) containing PRCGVPD sequence of the propeptide domain to form a glutathione S-oxide (GS(O)SR). S-glutathiolation of the propeptide inhibits its ability to coordinate and shield the catalytic Zn^{2+} ion, thus, an active proMMP is formed. This active proMMP may be further processed by other proteinases which can cleave the propeptide domain. (Figure based on work from References 47-49.)

Mechanistically, it has been shown that as little as 1-20 μM peroxynitrite, one of the most powerful endogenous oxidants formed by the diffusion limited reaction between nitric oxide and superoxide, causes S-glutathiolation of a sequence within the propeptide.[49] This S-glutathiolation takes place via disulfide S-oxide formation and produces a modification in size which is too small to detect by regular SDS-PAGE but can be detected using mass spectroscopy. Such a process, if it occurs intracellularly, could lead to inappropriate activation and proteolysis of proteins by MMPs within the cell. MMP-1, -2, -8, -9, have been shown to be activated by peroxynitrite in this way[47-49] although it has not been specifically tested whether other MMPs can also be activated in this manner.

Generally, the activation of MMPs by peroxynitrite (or other oxidants) occurs without the loss of the propeptide domain. This suggests that commonly used nomenclature which labels an MMP as being a 'proMMP' only by virtue of its higher molecular weight is both inaccurate and misleading. Such nomenclature does not take into account the potential for the higher molecular weight form of MMPs to be proteolytically active during oxidative stress, a condition common to several cardiovascular pathologies.

1.1.3 Inhibition of MMPs: TIMPs and chemical inhibitors

The tissue inhibitors of matrix metalloproteinases (TIMPs) provide another level of proteinase regulation by complexing with these MMPs and inhibiting their activity. Four TIMPs have been identified thus far and each binds to MMPs in a 1:1 stoichiometric ratio.[50] Although there is some binding preference of TIMP-2 with MMP-2, and TIMP-1 with MMP-9, the TIMPs in general do not show a high degree of specificity for any one MMP.[50] Indeed, there is no one study which has thoroughly examined the comparative inhibitory activity of all known TIMPs to a particular MMP. Structurally, TIMPs are two-domain molecules having an N-terminal MMP inhibiting domain and a smaller C-terminal domain. Three disulfide bonds stabilize each of these domains.[51] All four TIMPs have been observed in cardiac myocytes [4] and a variety of vascular cell types.

TIMP-1 and TIMP-2 are the best characterized TIMPs and they inhibit all known MMPs. TIMP-2 is constitutively expressed in a variety of cells of the heart, while TIMP-1 mRNA expression can be increased in response to signals such as cytokines in myocytes.[4] Both TIMP-1 and -2 also have diverse actions which are unrelated to inhibition of MMP activity, such as growth stimulating effects on erythroid precursor cells.[52,53]

TIMP-3, unlike the other TIMPs, binds tightly to the extracellular matrix and is usually difficult to extract from tissues.[50,54] High-levels of TIMP-3 transcripts have been detected in rat kidney, lungs, and heart.[55] Recently, TIMP-3 has been found to play an important role in inhibiting angiogenesis by attenuating the binding of vascular endothelial growth factor to its receptor.[56] Surprisingly, this effect was independent of its MMP inhibitory activity, since other TIMPs and synthetic MMP inhibitors could not inhibit binding of the growth factor to its receptor. Future studies will undoubtedly uncover other novel biological actions for other TIMPs which are independent of their MMP inhibitory action.

TIMP-4 appears to be the most abundant of the TIMPs in the myocardium – and in fact the heart and the brain are the only organs where abundant transcripts for TIMP-4 have been found.[5] With this pronounced expression in the cardiovascular system, it has been suggested that TIMP-4 protects against cardiomyopathy, tumor development, and metastasis.[12] It may also protect against vascular injury and remodeling, since TIMP-4 was found to be acutely upregulated in rat carotid arteries following experimental vascular damage.[57] An investigation of TIMP-4 from the Schulz lab has also localized this TIMP to the sarcomeres of rat ventricular myocytes, thus it may also play an important regulatory role in cardiomyocyte homeostasis.[58]

Aside from the natural endogenous inhibitors of MMPs, a number of synthetic inhibitors of MMPs are used in the laboratory and clinical settings. The tetracycline-class antibiotics are likely the most well recognized MMP inhibitors. The MMP inhibiting ability of these drugs is an additional effect independent of their antibiotic actions.[59] This has been demonstrated by studies which have chemically modified tetracyclines in a manner that resulted in a loss of antimicrobial activity but did not abolish its MMP inhibitory activity.[60] Doxycycline is widely used as it is one of the more potent MMP inhibitors of the tetracycline family and it is also better absorbed and has a longer half-life than the parent compound, tetracycline. *In vivo* and *in vitro* doxycycline has been demonstrated to potently inhibit MMPs.[61,62] It is believed that doxycycline inhibits MMPs by virtue of its ability to chelate divalent cations, and thus its ability to bind and inactivate the Zn^{2+} located at the catalytic site.

A number of proprietary compounds have also been developed by drug companies primarily as potential anti-arthritic and anti-cancer drugs. They share the common characteristic in having high specificity in binding to and inactivating the active site of MMPs. The hydroxamic-acid based inhibitors (e.g. GM6001, Ro31-9790) inhibit

MMPs by binding to the catalytic site through direct ligation of the catalytic Zn^{2+}, along with hydrogen bonding and van der Waals forces.[63,64] Use of these MMPs inhibitors has proven very effective in discovering novel roles of MMPs in both physiological and pathological processes. Unfortunately, their successful use in the clinical field has been limited, in part due to their side-effects (e.g. severe muscular pain).[65]

1.1.4 MMP-2, MMP-9, and TIMPs in chronic cardiovascular pathology

A variety of MMPs have been implicated in long term cardiovascular remodeling. For the purposes of this chapter, focus will be placed on the gelatinases since they are abundant in a variety of cardiac cells and a number of novel and acute functions have been elucidated for these MMPs. The gelatinases, MMP-2 (72 kDa and 62 kDa) and MMP-9 (92 kDa and 84 kDa), have been described in cardiac myocytes,[2] cardiac fibroblasts,[9] endocardial cells,[13] as well as endothelial[29] and vascular smooth muscle cells[18] (Table 1.1). MMP-2 is usually regarded as a constitutive MMP which is ubiquitously expressed throughout the body[66] whereas MMP-9 is often viewed as a cytokine inducible MMP.[67]

Peterson and colleagues found that MMP-2 activity is increased in the myocardium of spontaneously hypertensive heart failure rats compared to that of normotensive control rats. Interestingly, this increase corresponded with ventricular dilation and dysfunction as the animals aged, and the inhibition of MMP-2 activity by a four month treatment with a broad spectrum inhibitor could ameliorate remodeling and dysfunction.[68] Lee and colleagues demonstrated that targeted deletion of MMP-9 attenuated left ventricular remodeling after experimental myocardial infarction in mice.[69] In this study, the importance of MMP-9 in ventricular remodeling was highlighted by the fact that less dilation occurred in these knockout mice even though other MMPs were upregulated. Other evidence for the importance of MMP-2 and -9 in the heart was provided by a demonstration that selective MMP inhibitors (i.e. an MMP-1 sparing inhibitor) could ameliorate ventricular dysfunction in a model of heart failure in pigs caused by rapid ventricular pacing.[70]

MMP-2 and MMP-9 also play crucial roles in the development of long-term vascular pathologies. For instance, MMP-2 and MMP-9 are synthesized in large quantities by in rat carotid arteries following balloon catheter injury.[19] The increased expression and activity of these enzymes was followed closely by smooth muscle migration into the intima of these vessels. This study, along with a number of other investigations, have shown that inhibition of MMPs can lead to decreased neointimal formation in artherosclerosis.[71,72] Moreover, overexpression of MMP-9 can enhance vascular smooth muscle migration and advance vascular remodeling following arterial injury. [73,74]

MMP-2 and MMP-9 also play crucial roles in human cardiovascular disease. It has been found that the activities of both these MMPs were increased in the ventricles of patients suffering from dilated cardiomyopathy.[75] Specifically, MMP-2 activity was increased in the hearts of patients with non-ischemic dilated cardiomyopathy while MMP-9 activity was increased in both ischemic and non-ischemic dilated cardiomyopathy. In a recent study the concentration of plasma MMP-9 was identified as a novel predictor of adverse cardiovascular events.[76] Patients who experienced a fatal cardiovascular event were found to have higher concentrations of circulating MMP-9. Moreover, a particular polymorphism of MMP-9 (R279Q) was found to be highly associated with patients suffering from stable angina. Thus, several lines of evidence underscore the importance of MMP-2 and -9 in long term cardiovascular pathology.

A number of studies have also demonstrated that dysregulation of TIMPs also contributes to long term cardiovascular pathologies. The deletion of TIMP-1 in mice was recently found to potentiate adverse remodeling following experimental myocardial infarction.[77] In these mice ventricular weight and cross-sectional area of left ventricular myocytes were significantly increased, indicative of a pronounced hypertrophic response. In addition, fibrillar collagen content was reduced and myocardial infarct length was increased, which led the authors to conclude that TIMP-1 is important in myocardial structural remodeling. In a study of rats subjected to balloon injury of the carotid arteries, TIMP-4 was found to be acutely increased in the vascular adventitia within 24 h after injury.[57] One to two weeks after the injury TIMP-4 was found to be expressed throughout the layers of the vascular wall. Thus, dysregulation of TIMPs likely plays a role in cardiovascular pathology.

Nonetheless, a clear understanding of the role of TIMPs in these pathologies remains elusive. This is best evidenced by the conflicting data produced by studies of humans with ischemic and non-ischemic dilated cardiomyopathy. In one study, myocardial TIMP-1 and –2 protein content increased five times in dilated cardiomyopathy as compared to control patients[78] while another study revealed no differences in TIMP-1 content.[75] In contrast, Li *et al* found a decrease in TIMP-1 and –3 protein content and no change in TIMP-2 and –4 protein content,[79] while Rouet-Benzinab *et al* found both TIMP-1 and –2 were decreased in patients with dilated cardiomyopathy.[80] In patients with end stage congestive heart failure, TIMP-1, -2, and –

4 protein content was no different from control patients, but TIMP-3 content was significantly decreased.[81] From these conflicting results it is evident that further research of TIMPs in the cardiovascular field is necessary. One promising result relates to the fact that chronic unloading of the ventricle through the use of ventricular assist devices was associated with an increase in TIMP-1 and –3 content and a reduction in chamber dilation.[82]

1.1.5 Novel roles for MMP-2 and MMP-9

Given the abundant evidence linking MMPs with chronic cardiovascular disease, most researchers have focused on the long term proteolytic effects of MMPs on extracellular matrix substrates (i.e. collagen breakdown). Nonetheless, a number of novel acute effects for MMPs (on a seconds to minutes timescale) have been uncovered in the past few years. For instance, in a seminal investigation Sawicki *et al* demonstrated that MMP-2 is released by activated platelets and promotes platelet aggregation.[33] Moreover, exogenously added MMP-2 could stimulate aggregation while recombinant TIMP-2, as well as neutralizing antibodies against MMP-2, could prevent it. Further work has shown that MMP-1 also contributes to platelet aggregation through 'outside-in' signaling pathways.[32] In other investigations, MMPs were found to be involved in cell-to-cell signaling through the cleavage of chemokines. Overall and colleagues demonstrated that MMP-2 could cleave monocyte chemotractant protein-3 to an inactive peptide which acts as a chemokine receptor antagonist and dampens inflammation.[83]

MMPs also appear to acutely regulate vascular tone since MMP-2 can cleave big endothelin (ET) to yield the novel vasoconstrictor ET-1[1-32].[84] Further investigations found that MMP-9 could also produce this potent vasoconstrictor, and that ET-1[1-32] could promote neutrophil adhesion to endothelial cells.[85] MMP-2 was also shown to cleave and inactivate the vasodilator calcitonin gene related peptide.[86] Thus, in conditions where MMP-2 activity is upregulated, increased vascular tone may result from the combined activation of a vasoconstrictor propeptide and the inactivation of a vasodilator peptide.

These studies suggest that MMPs have as-yet-unknown regulatory actions in both normal and pathological conditions. Moreover, the word "matrix" in MMPs does not accurately reflect the full spectrum of their biological activities. In seeking a role for MMPs in acutely regulating cardiovascular function, we have investigated whether MMPs contribute cardiovascular dysfunction in a.) endotoxemia and b.) ischemia-reperfusion injury.

1.2 Septic shock and endotoxemia

1.2.1 Significance and definitions

The word "sepsis" is originally derived from the Greek language – to the Greeks, 'pepsis' embodied the natural process of maturation and fermentation, whereas 'sepsis' was synonymous with putrefaction.[87] Although our modern perception of sepsis is equally as negative, scientists and clinicians now define sepsis as the systemic host response to infection. At least 800,000 North Americans develop sepsis each year, and approximately one third of these die within 28 days of its onset.[88] As result of this mortality rate, sepsis remains the chief cause of death and disability in intensive care units, and it rivals myocardial infarction and almost all common cancers in terms of annual mortality.

There is great confusion and lack of standardization for the diagnosis of sepsis. Part of the confusion arises from the inconsistent nomenclature used in the literature to describe sepsis and associated problems. In order to establish working definitions which all scientists and clinicians should adhere to, the first International Sepsis Definitions Conference in 1992[89] (later revised in 2001)[90] agreed to several definitions. "Sepsis" is a syndrome characterized by both infection and systemic inflammatory response. This inflammatory response is defined by the presence of at least two of signs/symptoms defined in Table 1.2. "Severe sepsis" is sepsis complicated by severe organ dysfunction (e.g. renal failure). "Septic shock" is sepsis with circulatory failure characterized by persistent arterial hypotension. Along with sepsis a number of other conditions, such as acute respiratory distress syndrome and systemic inflammatory syndrome, are also often characterized as 'sepsis-like' syndromes due to their underlying inflammatory nature.

Table 1.2 Partial criteria for diagnosis of sepsis

Infection (documented or suspected)	
General Variables	fever (>38.3 °C) heart rate (>90/min) tachypnea altered mental status
Hemodynamic Variables	arterial hypotension (mean arterial pressure <70mmHg systolic blood pressure <90 mmHg) elevated cardiac index
Inflammatory Variables	leukocytosis or leukopenia elevated plasma C-reactive protein
Organ dysfunction Variables	arterial hypoxemia acute oliguria

(adapted from Ref. 90)

To date, treatment of septic shock is limited to antibacterial agents,[91] and standard therapies (e.g. fluid resuscitation) to manage the manifestations of this condition. Since septic shock is an inflammatory condition, most research has focused on the mediators of this inflammation. In the following sections, septic and endotoxemic cardiovascular dysfunction will be reviewed, the inflammatory cascade surrounding this condition will be discussed, and an overview of MMP involvement in sepsis and endotoxemia will be provided.

1.2.2 Cardiovascular manifestations of septic shock and endotoxemia

The direct correlation between poor cardiovascular function and poor prognosis highlights the importance of understanding the cardiovascular dysfunction associated with septic shock.[92-95] Wilson *et al* in 1965 were the first group to accurately portray the cardiovascular manifestations presented by approximately 90% of patients suffering from septic shock.[96] This profile is generally characterized by an elevated cardiac output, reduced afterload or systemic vascular resistance, and low preload (the exact state often depends on the level of fluid resuscitation).[97] A similar profile is also demonstrated in human volunteers administered LPS.[98]

Intrinsic myocardial dysfunction is a hallmark of septic shock and contributes to the cardiovascular manifestations of this condition. Calvin *et al* first demonstrated that left ventricular ejection fraction (<0.45 in most patients) is decreased despite increases in left ventricular end diastolic volume.[99] Typically, this dysfunction was sustained for 4 days and then function returned to normal in 7 to 10 days in those that survived. This observation of a reversible contractile depression was later supported by many other investigations.[100-102] Interestingly, however, this myocardial depression may in some way be adaptive, since nonsurvivors of septic shock typically maintain normal cardiac volumes and ejection fractions.[99,100]

In addition to myocardial dysfunction, systemic vasodilation produces a number of cardiovascular symptoms of septic shock. First, vasodilation reduces resistance to forward flow from the heart, and this allows cardiac output to reach supranormal values (effectively 'masking' the underlying cardiac dysfunction). In the later stages of septic shock, vasodilation and capillary leak combine to diminish the amount of blood returning to the heart, and this results in reduced ventricular filling pressures. Finally, vasodilation prevents resistance from being adequately high in microvascular circuits to properly perfuse organs, and this contributes to multiple organ dysfunction seen in severe sepsis.

In order to combat the systemic vasodilation seen in septic shock, high doses of intravenous epinephrine and norepinephrine are necessary to maintain blood pressure at an acceptable level.[103] The lack of reactivity to these pressor agents has never been fully explained, however, it has been proposed that this resistance is caused by either alterations in receptor abundance and affinity, or in defects in the intracellular signaling cascade. Alternatively, an overproduction of endothelium derived vasodilatory agents may also contribute to vascular hyporesponsiveness.[104] This latter aspect is discussed in Section 1.2.4.

1.2.3 Endotoxemia as a model of septic shock

In order to identify new modifiers of the cardiovascular dysfunction underlying septic shock, a readily accessible and reproducible model of this disease is necessary. One such model is endotoxemia, in which either human volunteers[98] or lab animals are injected with endotoxin.[105,106] Since its original description by Pfeffer and Koch in 1892, endotoxin, also called lipopolysaccharide (LPS), has been thought to play an important role in the pathogenesis of septic shock. LPS is a normal cell wall component of gram negative bacteria (e.g. *Escherichia coli, Salmonella typhosa*) which is shed periodically during a bacterium's life cycle and on lysis at death. There are three major regions of the LPS molecule: a) the repeating polysaccharide side chain which gives serotype specificity, b) a core oligosaccharide region, and the lipid A component.[107] The most significant of these structures is the lipid A portion, which is structurally consistent between different strains of gram-negative bacteria and appears to stimulate the inflammatory response in mammals.

In sepsis caused by gram-negative bacteria, LPS is carried through the blood by a specific protein (LPS binding protein) and interacts with CD14 receptors in the immune system.[108,109] The LPS-CD14 complex is then recognized by toll like receptor-4, a receptor found on macrophages, neutrophils, and endothelial cells that acts as an interface between the microbial and mammalian worlds.[110,111] As a family, the toll like receptors are known as pattern-recognition receptors due to their ability to bind to stereotypical and highly conserved biochemical structures that usually identify a molecule as being foreign and threatening.[112] Once a toll like receptor is occupied, it activates multiple signal transduction pathways and mobilizes a number of transcription factors (such as NF-κB[113]) which subsequently stimulate *de novo* transcription of hundreds of genes (e.g. proinflammatory cytokines, described in the next section).[114,115] Emerging work from toll like receptor field suggests the initiation of the septic response does not rely on the presence of viable microorganisms, but only the presence of molecules which the immune system recognizes as foreign. Thus, it follows that the injection of LPS alone into an animal (or human) would produces a septic-like syndrome which would serve as a good model of septic shock.

When LPS is administered to animals it mimics many of the hemodynamic, metabolic, and hematological manifestations of septic shock. The cardiovascular effects of LPS have been especially well characterized, and LPS has been demonstrated to reproduce the hypotension, vascular hyporesponsiveness, cardiac dysfunction, and systemic cardiovascular collapse seen in true septic shock.[116-118] When human volunteers are intravenously administered LPS (nanograms per kilogram body weight) many of the same signs and symptoms of sepsis are evoked.[98,119] In one reported case, a patient self-injected one milligram of LPS and this resulted in life threatening shock and organ dysfunction.[120] LPS also appears to be significant in true sepsis since pathologic concentrations of circulating LPS have been found in these patients.[121-123] Although most septic patients have low LPS concentrations (<500 pg/mL), patients with higher concentrations are most likely to be in shock and more likely to succumb to the condition.[122,123] Moreover, the antibiotic polymyxin B which binds and chelates endotoxin has been demonstrated to significantly reduce circulating levels of endotoxin and is currently in a Phase III trial.[124]

The central (and perhaps dominant) role of LPS in sepsis has also been supported by a number of other non-clinical investigations. For example, Huang and colleagues exposed dendritic cells to whole *E. coli* bacteria or bacterial LPS and monitored gene expression using oligonucleotide mircroarray analysis.[125] Surprisingly, of the 466 genes which were altered by whole *E. coli* almost 88% were also altered with LPS alone. Thus, despite the additional active molecules which are known to exist in bacteria, LPS alone was almost able to totally account for the full bacterial response. In another investigation, a lipid A-deficient strain of *Neisseria meningitidis* was developed.[126] When human monocytes were exposed to this mutant bacteria the production of pro-inflammatory cytokine production was significantly curtailed compared to the response to non-mutant bacteria.[127] These studies demonstrate that LPS is a fundamental signaling molecule in the inflammatory process associated with sepsis.

1.2.4 The contribution of inflammatory cytokines to septic shock

It is widely accepted that septic shock is the result of an inability to regulate the inflammatory response. This theory was first popularized by L. Thomas in 1972 when he stated that "the microorganisms that seem to have it in for us [are] more like bystanders…It is our response to their presence that makes the disease. Our arsenal for fighting off bacteria are so powerful…that we are more in danger from them than the invaders."[128] In other words, it is the inflammatory cascade which results from sepsis that ultimately produces the cardiovascular dysfunction seen in septic shock.

As discussed in the previous section, the inflammatory activation and amplification cascade is largely initiated through the body's interactions with components of the invading organism (e.g. LPS). Through this interaction a number of powerful pro-inflammatory cytokines such as interluekin-2 (IL-2), IL-4, IL-6, IL-8, IL-10, interferon-γ (INF-γ), and in particular tumor necrosis factor-α (TNF-α) and IL-1β, are produced in endothelial cells, neutrophils, macrophages, and lymphocytes.[129-134] Most of the manifestations of septic shock occur when this 'cytokine storm' is unleashed, with TNF-α and IL-1β playing principal roles in the inflammatory process.

TNF-α is a crucial early mediator of endotoxemia and septic shock.[135,136] After LPS administration to anesthetized dogs, myocardial depression coincides with peak TNF-α serum concentrations.[137] Moreover, several groups have shown that administration of TNF-α reproduces all the cardiovascular abnormalities of human septic shock in dose-dependent manner.[138-140] These abnormalities include hypotension, decreased cardiac output, ventricular dilation, and depressed ejection fraction.

In animal models it has been demonstrated that passive immunization against TNF-α protectes mice from lethal endotoxemia,[135] and that TNF-α blockade is beneficial against *E. coli* or LPS induced shock,[136,141] or *Staphylococcus aureus* induced shock in primates.[142] Clinically, circulating levels of TNF-α correlate with clinical outcome[130] and the risk of death among patients with septic shock has been linked with polymorphisms for TNF-α.[143] To date, over ten multicentre randomized trials of various anti-TNF antibodies have been completed.[124] Unfortunately, the benefits of this treatment were found to be modest at best, with a 3.5% overall reduction in 28 day mortality.[124]

IL-1β is another proinflammatory cytokine which is synthesized by toll like receptor 4 engagement[144] and has been shown to evoke similar pathophysiological responses as TNF-α.[145,146] This cytokine is elevated in both animal models of endotoxemic and septic shock,[117,147] and in human septic shock.[129] In experimental settings, infusion of this cytokine into animals and humans results in marked cardiac dysfunction and hypotension, and produces a number of metabolic disorders which also occur in septic shock.[146]

Blockade of IL-1β has also been found to exert beneficial effects. For instance, in animal models of endotoxemia, an IL-1 receptor antagonist was found to reduce cardiovascular dysfunction and mortality.[148-150] When this same approach was evaluated clinically in three separate trials the results were modest with a 4.9% decrease in mortality noted.[124] The failure of these anti-cytokine trials to produce greater reduction in mortality is likely due to the redundancy in effects of many cytokines. Since IL-1 and TNF-α have such similar effects[146] blocking one does not necessarily inhibit the downstream process as the other non-antagonized cytokine will stimulate the same process. Moreover, other pro-inflammatory cytokines that are expressed in septic shock likely have effects that overlap those of TNF-α and IL-1β.

An inflammatory view of septic shock is supported by the mechanisms of action of the two new therapies approved for treatment of this condition. First, low dose corticosteroids have proven an effective treatment in septic patients who are unresponsive to adrenocorticotropic hormone stimulation.[151,152] Corticosteroids have long been recognized to have potent anti-inflammatory effects, and serum from patients treated with these drugs had decreased TNF-α, IL-6 and IL-8 concentrations. As well serum from these patient also caused significantly less NF-κB activation and cytokine production in peripheral blood leukocytes.[152]

Second, activated protein C has been shown to reduce relative risk of death in septic shock patients by almost 20%.[124] Although this drug is most widely recognized for its ability to decrease disseminated intravascular coagulation, it should be noted that this drug is also anti-inflammatory as it blocks cytokine production from monocytes and blocks cell adhesion.[153] Moreover, the failure of other anticoagulants (antithrombin III and tissue factor pathway inhibitor)[124] underlines the importance of protein C's anti-inflammatory actions.

Nonetheless, the success of these two drugs is balanced by the clinical failure of scores of other anti-inflammatory agents. Trials targeting platelet activating factor, nitric oxide production, bradykinin, and arachidonic acid metabolites have all failed to significantly reduce mortality in septic patients.[124] Although our understanding of the fundamental biology of septic shock has rapidly advanced, translating these insights into new therapies has proven nearly impossible. Part of this failure is likely due to the complex nature of the septic inflammatory response – with so many mediators (many of which are redundant) it is not possible to adequately treat the condition by targeting upstream mediators. Instead, more end effectors of septic shock need to be identified and targeted.

1.2.5 Nitric oxide and peroxynitrite as effectors of septic cardiovascular dysfunction

Hundreds of downstream effectors of proinflammatory cytokines have been identified, however, one effector that has received a great deal of attention over the past 15 years is nitric oxide (NO). NO is a labile gas with a half-life of a few seconds at 37 °C in the biological milieu. NO is generated by three isoforms of nitric oxide synthase (NOS), endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS. Under normal physiological conditions NO is primarily generated in the cardiovascular system by eNOS in cardiac myocytes, endocardial cells,[154] and vascular endothelial cells.[155] eNOS catalyzes the five-electron oxidation of L-arginine to produce NO and a by-product, citrulline. This catalytic activity is tightly regulated by intracellular calcium levels and, as a result, only small quantities of NO are produced for brief periods when intracellular Ca^{2+} levels are elevated. *In vivo*, this low level of NO production exerts a number of regulatory and cytoprotective effects: a) decreasing intracellular calcium levels by increasing cyclic GMP production;[156] b) promoting vasodilation;[157] c) decreasing adhesion of platelets[158] and neutrophils[159] to the endothelium; and d) regulating cellular metabolism by reversibly inhibiting mitochondrial respiration[160] and enzymes involved in glycolysis.[161]

NO can also be produced in high concentrations under conditions of inflammatory stress following the expression of inducible NOS (iNOS) in endocardial endothelium,[154] vascular endothelial cells,[162] cardiac myocytes,[105,163] vascular smooth muscle[164] and neutrophils.[165] Exposure of isolated human vascular smooth cells to a variety of cytokines (IL-1β, IFN-γ, TNF-α) induces iNOS.[166] Exposure of isolated rat hearts to these cytokines also induces iNOS expression in the myocardium.[167,168] iNOS, unlike eNOS, is independent of intracellular calcium levels and thus produces higher rates of NO formation which is sustained over several hours. Evidence of this enhanced NO production can be seen in septic patients as significantly elevated plasma levels of stable NO metabolites (NO_2^- and NO_3^-).[169,170]

An overproduction of nitric oxide likely contributes to the cardiac dysfunction and systemic vasodilation seen during sepsis and endotoxemia.[106,166,171,172] The potential relevance of NO in these conditions was supported by studies which demonstrated that NOS inhibitors could attenuate the adverse cardiovascular effects of TNF-α, IL-1β, or LPS administration.[173,174]

NO likely exerts these negative effects through the formation of peroxynitrite, the toxic reaction product of NO and superoxide anion (Fig 1.2). At the same time that NO is being produced in the endotoxemic cardiovascular system, large amounts of superoxide anion are also generated.[175] NO and superoxide anion then rapidly react to form peroxynitrite.[176,177] In a physiological CO_2/bicarbonate environment peroxynitrite then reacts with CO_2 to form nitrosoperoxycarbonate anion ($ONOOCO_2^-$).[178-180] This molecule is then protonated at physiological pH to form nitrosoperoxycarbonic acid. Since this intermediate is highly unstable, it spontaneously decomposes by homolytic cleavage to give rise to nitrogen dioxide, hydroxyl radicals, and CO_2. Both nitrogen dioxide and hydroxyl radicals are far more chemically reactive than peroxynitrite and thus represent the actual species responsible for the detrimental effects of peroxynitrite.

The targets of peroxynitrite in the cell include proteins,[181] lipids,[182] carbohydrates,[183] and nucleic acids.[184] The reaction of peroxynitrite with these biomolecules results in lipid peroxidation,[182,185] protein modification by oxidization of sulfhydryl groups[186] and nitration of tyrosine residues.[187] The detrimental effects of peroxynitrite include structural damage, enzyme dysfunction, ion channel and transporter malfunction and eventually cell death. These negative effects of peroxynitrite likely contribute to septic cardiovascular dysfunction since evidence of its production can be found in septic and endotoxemic humans and animals.[106,188,189] Using immunohistochemical techniques, intense nitrotyrosine staining has been detected in the endocardium, myocardium, and coronary vascular endothelium of cardiac tissue obtained from septic patients.[25]

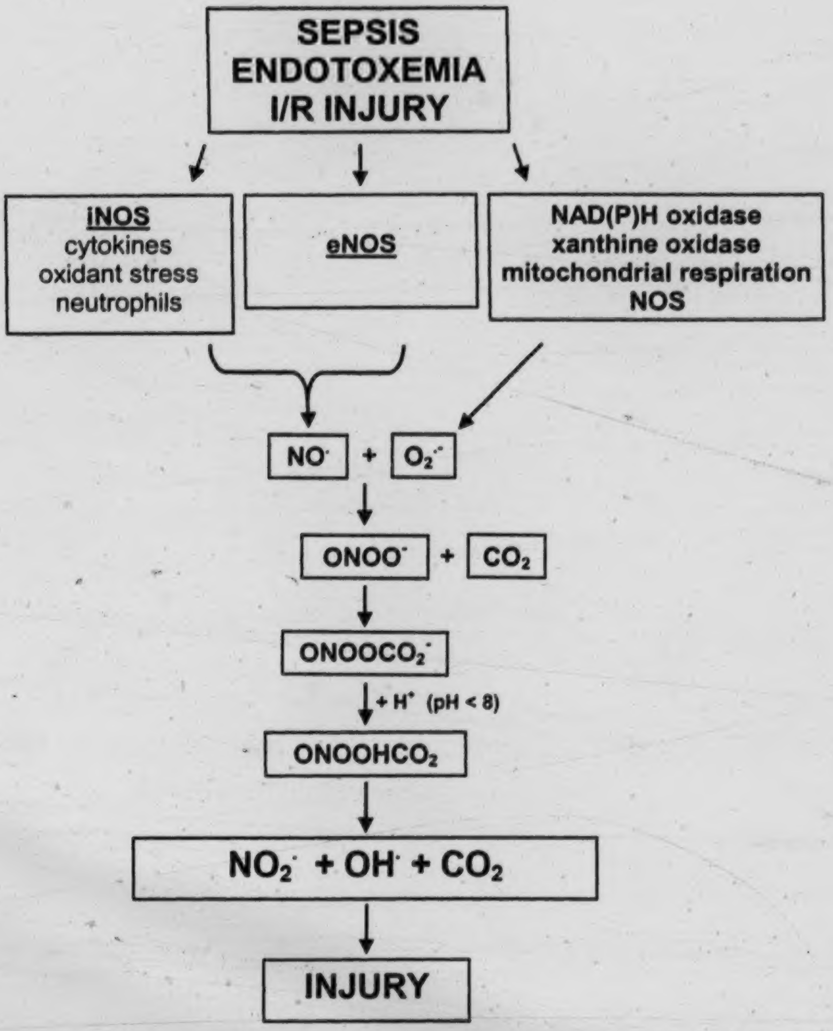
SEPSIS
ENDOTOXEMIA
I/R INJURY
iNOS
cytokines
oxidant stress
neutrophils
eNOS
NAD(P)H oxidase
xanthine oxidase
mitochondrial respiration
NOS
NO· + O₂·⁻
ONOO⁻ + CO₂
ONOOCO₂⁻
+ H⁺ (pH < 8)
ONOOHCO₂
NO₂· + OH· + CO₂
INJURY

Figure 1.2 Peroxynitrite formation. The inflammatory stimulus in conditions such as sepsis, endotoxemia, and ischemia-reperfusion (I/R) injury induces NO production (from either eNOS and/or iNOS) and $O_2^{\cdot -}$ production (from NAD(P)H oxidase, xanthine oxidase, uncoupled mitochondrial respiration, and NOS under conditions of L-arginine or tetrahydrobiopterin depletion). These two molecules react to form peroxynitrite ($ONOO^-$), which then adds carbon dioxide to form nitrosoperoxycarbonate anion ($ONOOCO_2^-$). This moiety is protonated at physiological pH to form nitrosoperoxycarbonic acid ($ONOOHCO_2$) which is highly unstable and rapidly decomposes into highly toxic free radical species. (Adapted from Ref. 190)

These studies have clearly demonstrated that NO overproduction plays a large role in the cardiovascular dysfunction associated with septic shock. However, it should be noted that mice lacking iNOS, although unable to increase their nitrate production, are not protected against lethal doses of LPS.[191,192] These results can be explained in part by the beneficial cytoprotective effects that NO exerts, as well as by the bacteriocidal effects of peroxynitrite in true sepsis. Thus, although NO blockade may be beneficial to some degree, it is likely that NO inhibition will never become a therapeutically viable option due to the beneficial effects of NO in sepsis. In order to overcome this difficulty, the negative effectors of peroxynitrite toxicity in sepsis need to be identified. Since peroxynitrite activates MMPs (discussed in Section 1.1.2), and cytokines can also regulate the expression of these proteinases,[4,18] it is likely that MMPs play a role in septic shock.

1.2.6 MMPs in endotoxemia and septic shock

Although few investigations have been carried out examining MMPs in sepsis, a number of observations suggest that these proteinases contribute to this disease. This section will briefly overview the studies which have linked MMP activation with endotoxemia and septic shock. Evidence has been provided using isolated cell culture models, whole animal models, humans volunteers administered LPS, and septic shock patients.

Using human umbilical vein endothelial cells, Kim *et al* investigated the effect of growth factors (vascular endothelial growth factor, fibroblast growth factor, angiotensin I and II, hepatocyte growth factor), cytokines (TNF-α), and LPS on MMP activity. [193] Only MMP-2 activity could be seen in these cells, and interestingly only LPS was found to increase MMP-2 activity during the 3 h incubation period. Through the use of selective pharmacological inhibitors it was demonstrated that this activation was dependent on an NF-κB pathway. LPS was found to have similar effects on isolated murine macrophages by Xie *et al.*[194] In these cells both MMP-2 and MMP-9 secreted activities could be detected, and the addition of LPS increased the activity of both MMPs in a concentration-dependent manner. A time course analysis of activities demonstrated discordant kinetics between the two MMPs, since MMP-9 was stimulated within 1 hour of LPS while MMP-2 activity was increased after 16 h. Pugin *et al* performed similar experiments using whole human blood.[195] In this model, LPS had no effect on MMP-2 activity but increased MMP-9 activity within 0.5 h of LPS stimulation. Neutrophils were later demonstrated to be the source of increased MMP-9 activity. Overall, cell culture experiments (although somewhat conflicting) support the notion that LPS can stimulate MMP-2 and –9 activities.

In animal models of endotoxemia and septic shock MMP activation has also been shown. In a baboon subjected to *E. coli* induced sepsis, circulating MMP-9 was found to be increased between 2 to 4 h post bacterial injection.[196] Pagenstecher *et al* injected mice with LPS and monitored MMP gene expression in the liver, spleen, and kidney.[197] In this model MMP-9, MT1-MMP, stromelysin-3, and collagenase 3 mRNA were found to be elevated in all three organs at various time points following LPS injection. At the same time, TIMP-1 and TIMP-3 mRNA were also found to be elevated. MMP-9 activity was also increased in all three organs, while MMP-2 activity remained unchanged. Using *in situ* zymography, it was demonstrated that net gelatinolytic activity was increased in the organs studied (thus, even though TIMP gene expression was increased there was still a net increase in MMP activity).[198] Following up on this study, the same group also investigated the effects of LPS on a MMP-9 knockout mouse.[198] Deletion of MMP-9 gene did not result in compensatory upregulation of any other MMP mRNA (nine were measured), and it was found that these mice were significantly more resistant to lethal doses of LPS.

The potential involvement of MMP in LPS induced cardiovascular dysfunction was partially addressed by Carney *et al.*[199] Using a swine model, blood pressure was monitored in control, LPS infused, and LPS + MMP inhibitor infused animals. As expected, LPS significantly reduced blood pressure when compared to control animals. Intriguingly, the LPS + MMP inhibitor treated group did not suffer a similar loss in blood pressure. Several shortfalls exist in this study, however, as changes in individual MMPs were never examined in this study, and the target(s) of the MMP inhibitor was never identified.

Other studies have examined changes in MMPs during endotoxemia and sepsis in humans. Radomski and colleagues administered low doses of LPS in human volunteers and found that circulating MMP-9 activity (but not MMP-2 activity) significantly increased within 1 h.[119] This increase was accompanied by a significant decrease in blood pressure. Pugin *et al* conducted a similar study and found that the LPS induced increases in MMP-9 activity could be partially blunted by coadministration of a TNF-α receptor antagonist.[195] In this same study two septic patients were studied and it was shown that both MMP-9 and MMP-2 circulating activities were significantly increased, and that the levels of MMP activity paralleled the severity of clinical condition. A potential role for MMP-9 was confirmed in a separate study which demonstrated that circulating MMP-9 protein levels were significantly higher in non-survivors than survivors, and that MMP-9 protein levels correlated with septic severity.[200] A connection between MMP-9 and LPS was also provided in this study since MMP-9 levels correlated with LPS concentration, and decreasing circulating LPS levels using polymyxin B also significantly decreased MMP-9 as well.[200]

Taken as a whole, these studies provide a starting point for further investigations into the role of MMPs in endotoxemia and septic shock. In whole animal models, the activity of MMPs in the cardiovascular system has yet to be described. As well, the effects of MMP inhibition on the cardiac and vascular dysfunction also remain unknown. These issues will be explored in my thesis.

1.3 Myocardial ischemia-reperfusion injury

1.3.1 History and definition

The first description of myocardial ischemia-reperfusion (I/R) injury was made in 1975 when Heyndrickx *et al* observed persistent regional myocardial mechanical dysfunction after brief coronary ligation and reperfusion in dogs.[201] Braunwald and Kloner[202] later named this phenomenon myocardial 'stunning' injury. Clinically, I/R and stunning injuries are seen with the use of thrombolytics following infarction[203] and also as a result of surgery involving cardiopulmonary bypass.[202] The pathogenesis of I/R injury in the heart includes several mechanisms that have been intensively studied in the recent years.[204] These include damage caused by: a) alterations in cardiac metabolism,[205] b) the production of reactive oxygen species,[177] c) alterations in calcium handling,[206] and, most recently, d) the intracellular activation myocardial MMPs. The following sections discuss the inflammatory aspects of I/R injury and then review the investigations which have implicated MMPs in this pathology.

1.3.2 I/R injury as a condition of inflammatory stress

I/R injury can be considered a condition of inflammatory stress in part by its association with oxidative stress and cytokine production. Despite the absence of oxygen supply to the myocardium during ischemia, the production of superoxide anion, hydrogen peroxide, and hydroxyl radical is enhanced.[207] Upon reperfusion, the reintroduction of oxygen after ischemia leads to an even higher generation of reactive oxygen species and a large concentrated burst of NO which can be measured by electron spin trap techniques.[176] At the same time that NO is being produced, large amounts of superoxide anion are also generated.[176,208] Possible sources of superoxide anion are NAD(P)H oxidase, xanthine oxidase,[209-211] uncoupled mitochondrial respiration,[212] and nitric oxide synthase itself if there is a lack of either its cofactor tetrahydrobiopterin or its substrate L-arginine.[213-215] NO and superoxide anion rapidly react during early reperfusion to form peroxynitrite.[176,177,216] which then reacts with a number of different proteins (discussed in Section 1.2.4) and contributes to decreased function in the post-reperfused myocardium.

Along with peroxynitrite formation, cytokine production has also been demonstrated to play a pivotal role in myocardial I/R injury. For instance, following I/R injury in crystalloid perfused rat hearts, TNF-α protein is elevated in the reperfused myocardium.[217,218] IL-1β, IL-2, IL-6, IL-8 are other important cytokines which have also been demonstrated to be increased following I/R injury.[219-224] These cytokines likely play an important role in the cardiac dysfunction following reperfusion, and other studies have clearly shown that TNF-α,[225,226] and a mixture of cytokines (IL-1β, IFN-γ, and TNF-α)[167,168] have cardiodepressant effects.

Clinically, a number of studies have demonstrated an increase in inflammatory stress following I/R. In a study of patients who underwent successful cardiopulmonary resuscitation (a form of systemic I/R), it was found that many of the same inflammatory markers which are elevated in sepsis were also elevated following this intervention. These include elevations in IL-6, IL-8, IL-10, and soluble TNF-α receptor II.[227] Similar elevation have also been noted in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass. In this surgery, the heart is subjected to a type of global, no-flow ischemia which results in a well-characterized reversible cardiac dysfunction.[228-230] Myocardial peroxynitrite formation is increased and studies have shown that a number of cytokines (TNF-α, IL-1β, IL-2, IL-6, and other proinflammatory cytokines) are increased following this surgery.[231-235] As well, circulating endotoxin concentrations are also increased and this would likely contribute to a proinflammatory cascade.[236-240] Thus, overall inflammation is increased following I/R injury, and this produces an immunological profile similar to that found in patients with sepsis and animals subjected to experimental endotoxemia.

1.3.3 Ischemic preconditioning ameliorates I/R injury

One intervention which has been demonstrated to decrease I/R injury induced inflammation is ischemic preconditioning. Ischemic preconditioning is a well-described adaptive response in which brief exposure to ischemia markedly enhances the ability of the heart to withstand subsequent ischemic injury. This phenomenon was first described by Murry *et al* when they observed that the size of infarct resulting from a 40 min occlusion of a canine coronary artery could be significantly reduced if the heart were subjected to four brief periods of 5 min of ischemia and 5 min of reperfusion immediately prior to the longer ischemic period.[241] The phenomenon of preconditioning has been shown to exist in every animal model tested, including rats,[242] rabbits,[243] and pigs,[244] and there is strong circumstantial evidence that this preconditioning also takes place in the human heart.[245-247]

The exact molecular and cellular mechanism of preconditioning is still a question of debate. In recent years, it has been suggested that opening of mitochondrial K_{ATP} channels is the end effector of all preconditioning stimuli.[248,249] However, it is still unclear why K_{ATP} opening would be protective and whether K_{ATP} channels are indeed the end effectors or simply act as another signal transduction step.[250] Regardless of the exact mechanism, preconditioning is the first intervention to unambiguously limit cardiac injury and mechanical dysfunction, likely through its ability to limit a number of inflammatory mediators such as peroxynitrite[251] and TNF-α[252] following I/R injury.

1.3.4 Role of MMP-2 in acute myocardial I/R injury

The acute activation of MMP-2 in myocardial I/R injury was first demonstrated by the Schulz lab in isolated rat hearts.[253] Hearts were excised, aerobically perfused, and then subjected to global, no-flow ischemia followed by 30 min of aerobic reperfusion. During periods of aerobic perfusion cardiac mechanical function was noted and samples of coronary effluent were collected. It was found that 20 min of ischemia significantly increased the release of MMP-2 activity into the coronary effluent upon reperfusion, and this increase actually peaked within the first 5 min of reperfusion. The increase in coronary effluent MMP-2 activity was coupled with a decrease in its activity in the ventricles. Thus, in the setting of I/R, the most abundant gelatinase in the rat myocardium was activated and released from the heart in a time frame of seconds to minutes.

The acute activation of MMP-2 was demonstrated to be functionally significant by several experiments.[253] Increasing the ischemic time was found to increase MMP-2 activation and release, and this increase in activity correlated inversely with the resulting cardiac dysfunction. In other words, increasing the ischemic insult correlated with greater MMP-2 activation, and greater MMP-2 activation was related with poorer cardiac function upon reperfusion. Infusion of a preparation of MMP-2 into the heart subjected only to a 15 min period of ischemia (insufficient in itself to cause stunning upon reperfusion) diminished the recovery of function during reperfusion. The recovery of cardiac function upon reperfusion was enhanced in a concentration-dependent manner by a neutralizing antibody against MMP-2, or with the use of MMP inhibitors such as doxycycline or o-phenanthroline.

A recent study confirmed the damaging role of MMPs in acute I/R injury. Prasan *et al* used isolated rabbit hearts to demonstrate that MMP-2 is activated and released during reperfusion following prolonged ischemia.[254] These results suggest that MMP-2 may contribute to myocardial dysfunction following prolonged ischemia in rabbit hearts.

Other studies have confirmed a role for MMPs in I/R injury *in vivo*. Romanic *et al* used an *in vivo* mouse model in which the lower anterior descending coronary artery was occluded for 30 min followed by 4 to 24 h reperfusion.[255] Following I/R injury myocardial MMP-2 activity was significantly increased and MMP-9 activity (associated with neutrophil infiltration) was also found. Selectively deleting MMP-9 decreased both the infarct size and neutrophil infiltration following I/R, with homozygous (-/-) mice showing greater protection than heterozygous (+/-) mice.

Mehta and colleagues have used an *in vivo* rat model of I/R in which the total left coronary artery was occluded for 60 min and then the heart was reperfused for 60 min.[256,257] Associated with the cardiac dysfunction upon reperfusion was a significant upregulation of MMP-1 protein within the myocardium. Pretreating these animals with either transforming growth factor-β_1 [256] or an antibody against lectin-like oxidized low-density lipoprotein receptor (LOX-1)[257] not only protected the myocardium, but also inhibited the upregulation of MMP-1. Moreover, pretreatment of adult rat myocytes with a broad spectrum MMP inhibitor (PD-166793) attenuated MMP-1 mediated inury.[256]

1.3.5 Consequences of MMP activation in I/R injury

Given the evidence for a net increase in MMP activity following I/R, proteolysis of susceptible target proteins is a potential mechanism by which MMPs cause diminished contractile function. Collagen and other extracellular matrix proteins are the most obvious targets. However, in the isolated rat heart model of I/R there is no evidence for collagen degradation in the time scale of 20 min of ischemia followed by reperfusion (Ref 258 and unpublished observations, Schulz lab). Indeed others have shown that protective actions of MMP inhibitors on myocardial contractile function following infarction were independent of changes in collagen content.[259] As extracellular matrix degradation does not fully explain the observed mechanical dysfunction other proteolytic targets for MMP-2 likely exist.

Proteolysis of myocardial sarcomeric and cytoskeletal proteins has been proposed as a mechanism of the cardiac dysfunction seen following I/R injury. In 1997, Marban and colleagues first proposed that the regulatory contractile protein troponin I (TnI) is degraded in an isolated rat heart model of brief ischemia (20 min) followed by 30 min reperfusion.[260] In the following years many investigators have confirmed this observation and the evidence for the involvement of TnI degradation (as well as its post-translational modification) in I/R injury is accumulating.[261] Van Eyk and colleagues identified one of the TnI fragments as TnI_{1-193} in isolated rat hearts subjected to a 60 min duration of ischemia.[262] In a subsequent study this fragment was overexpressed in mice, and the resulting transgenic mouse displayed depressed cardiac contractility which resembled features of I/R injury.[263] Moreover, in myocardial biopsy samples taken from patients undergoing cardiopulmonary bypass surgery, a scenario mimicking acute I/R injury, the proteolysis of TnI was also demonstrated.[262,263] Since the enzyme(s) responsible for the proteolysis of TnI is not clear, it was speculated that MMP-2 may contribute to the degradation of TnI during I/R.

Recombinant human MMP-2 was found to rapidly degrade recombinant TnI and TnC (but not TnT) *in vitro*, and also TnI alone when assembled in the intact troponin complex (consisting of TnI, TnC and TnT).[264] This degradation was inhibited by TIMP-2 and other synthetic MMP inhibitors. When isolated perfused rat hearts were subjected to 20 min global ischemia followed by 30 min reperfusion troponin I was found to be significantly degraded in the myocardium. Treatment of hearts with MMP inhibitors o-phenanthroline or doxycycline significantly improved the recovery of mechanical function and also protected against TnI degradation.

These results not only indicate that TnI may be a proteolytic target for MMP-2, but also suggest a novel intracellular locus of action for this MMP within the cardiac myocyte. Using immunogold electron microscopy it was shown that MMP-2 is found in close association within the sarcomeres of the cardiac myocyte. Immunofluorescent confocal microscopy confirmed this result, showing that MMP-2 colocalizes with troponin I.[264] Interestingly, when TnI was immunoprecipitated from myocardial homogenates MMP-2 activity was present in the precipitated complex. As well, MMP-2 activity could be found in a purified preparation of thin myofilaments (consisting of actin, tropomyosin, and TnI) prepared from ischemic-reperfused rat hearts, and the level of MMP-2 protein was enhanced after I/R injury. Thus, several lines of evidence suggest MMP-2 has an intracellular locus of action on TnI in mediating I/R injury.[264] Moreover, as described in Section 1.3.6, TIMP-4 was also localized to the sarcomeres by electron microscopy, and less TIMP-4 is present within the sarcomeres following I/R injury.[58]

Based on the findings of these studies, it is likely that MMP-2 was inappropriately activated during I/R injury and this, coupled with the loss of TIMP-4 from the sarcomere, produces a localized area of increased proteolysis within the cardiomyocyte (Fig 1.3). The activated MMP-2 cleaves TnI and the former is then rapidly released from the cardiomyocyte. In acute myocardial I/R injury, the rapid release of MMP-2 may act as a safety mechanism to protect the heart from further proteolytic stress.

Other immunohistochemical evidence exists which demonstrates an intracellular locus of MMPs in the cardiomyocyte. Using serial section confocal microscopy Rouet-Benzinab *et al* demonstrated that MMP-2 and MMP-9 are associated with the sarcomeres of cardiomyocytes in biopsies taken from patients with dilated cardiomyopathy.[80] Immunohistochemical evidence has also shown dense intracellular staining for MMP-2[265] and MMP-9[82,265] within human cardiomyocytes. Confocal microscopy demonstrated that

MT1-MMP and MMP-2 are associated with a sarcomeric banding pattern in isolated porcine left ventricular myocytes.[2,266] Finally, in a study of isolated human left ventricular myocytes using immunoflourescent staining, MT1-MMP was colocalized to the sarcomeric protein α-actinin.[75] Colocalization with this latter protein suggests that MMPs may also be responsible for α-actinin degradation seen following I/R.[267]

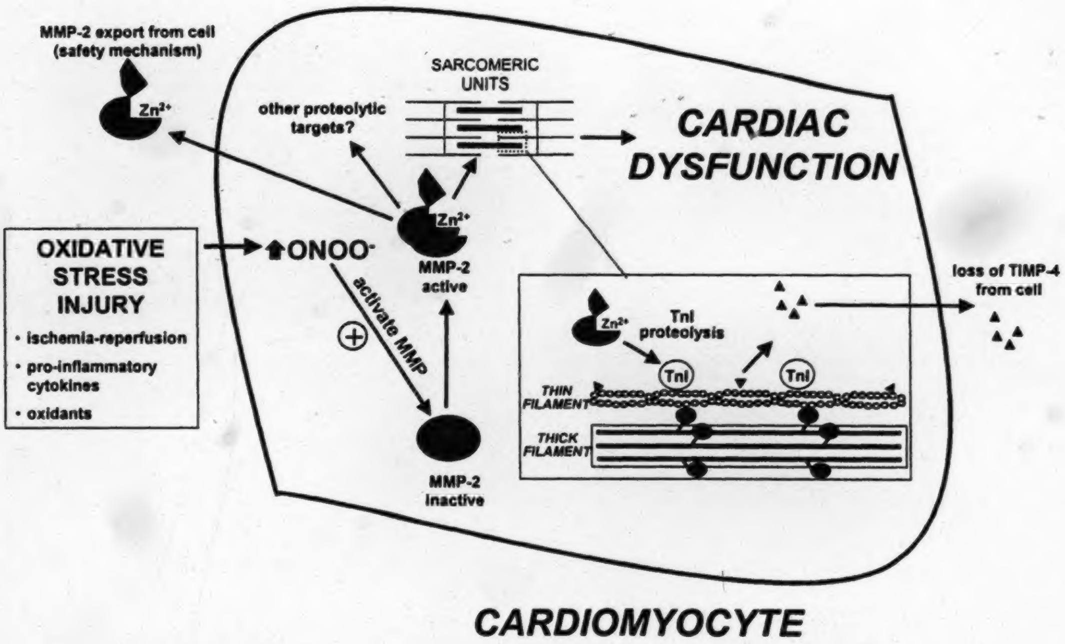
MMP-2 export from cell (safety mechanism)
Zn^{2+}
other proteolytic targets?
SARCOMERIC UNITS
CARDIAC DYSFUNCTION
OXIDATIVE STRESS INJURY
• ischemia-reperfusion
• pro-inflammatory cytokines
• oxidants
$ONOO^-$
activate MMP
⊕
MMP-2 active
MMP-2 inactive
TnI proteolysis
TnI
THIN FILAMENT
THICK FILAMENT
loss of TIMP-4 from cell
CARDIOMYOCYTE

Figure 1.3 Paradigm of MMP-2/TIMP-4 in oxidatively stressed cardiomyocytes: intracellular localization, inappropriate activation, and proteolysis of troponin I (TnI). Peroxynitrite ($ONOO^-$) production is increased during oxidative stress injuries (such as ischemia-reperfusion or insult by pro-inflammatory cytokines). Peroxynitrite activates MMP-2, forming an active enzyme with the prodomain still intact. Both MMP-2 and TIMP-4 are localized with thin myofilaments located in the sarcomere. The activation of MMP-2 coupled with the loss of TIMP-4 (▲) from the thin myofilaments produces a localized area of increased proteolysis within the sarcomere, and leads to troponin I degradation. The release of MMP-2 by the cell may be a means to limit the proteolytic stress. The net result of this inappropriate intracellular activation of MMP-2 is cardiac dysfunction.

1.3.6 TIMPs and I/R injury

Although evidence of MMP involvement in I/R injury is rapidly accumulating, little work has been done to examine TIMPs in this pathology. Considering that a dysregulation of TIMPs has been implicated in long term cardiovascular pathology a similar dysregulation may contribute to acute cardiac injury as well.

As described above, TIMP-4 is highly expressed in the cardiovascular system and is likely an important inhibitory regulator of MMPs in the heart.[5] In rat hearts subjected to 20 min of global, no-flow ischemia followed by 30 min of reperfusion there was a rapid release of TIMP-4 from the heart during the first minutes of reperfusion.[58] This release could be detected by both western blot and reverse zymography of the perfusate during reperfusion. Importantly, no other TIMP activity was measurable in the coronary effluent apart from TIMP-4. Immunogold electron microscopy of myocardial tissue revealed the association of TIMP-4 with the sarcomeres as well as a loss of TIMP-4 following I/R. Mirroring the release of MMP-2 described above, there was a negative correlation between the recovery of cardiac mechanical function and the release of TIMP-4 during reperfusion in hearts subjected to longer durations of ischemia. The loss of TIMP-4 resulted in a net increase in myocardial MMP activity as demonstrated using *in situ* zymography. Thus, although there is a loss of myocardial MMP-2 as a consequence of I/R, it appears that there may be a greater loss of inhibitory TIMP-4 activity and this results in a tip in the balance towards enhanced intracellular proteolytic activity (Fig 1.3).

Using isolated rabbit hearts subjected to 20 min of ischemia followed by 120 min reperfusion Baghelai *et al* found that the level of TIMP-1 mRNA was significantly depressed following reperfusion, although no functional data were reported in this paper.[268] Thus, in I/R a decrease in TIMP-1 may also favor enhanced MMP activity and a balance in favor of proteolysis.

1.3.7 Clinical I/R injury and MMPs/TIMPs

To date, very little information exists as to the status of MMPs within clinical settings of I/R. A well characterized, clinically relevant form of myocardial I/R injury occurs in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass. In patients undergoing this procedure, Mayers *et al* analyzed MMP activity in plasma and myocardial samples from patients prior to the ischemic insult (i.e. placement of the aortic cross clamp) and following reperfusion (i.e. release of the aortic cross clamp).[265] It was found that both MMP-2 and –9 activities were significantly increased at the termination of bypass in the reperfused myocardium, while only MMP-9 activity was increased in the plasma at this time. Biopsy samples were immunostained following reperfusion and MMP-2 and -9 were diffusely expressed in the cytoplasm of cardiac myocytes. Unfortunately, none of the activities were correlated with any type of clinical marker or outcome, and thus it is difficult to speculate as to the role of MMPs within this clinical setting of I/R.

1.3.8 Free radicals – a bridge uniting inflammation with MMP activation within the cardiovascular system

A hallmark of virtually all inflammatory processes is the generation of free radicals. Peroxynitrite, a potent free radical discussed in previous sections, has been found to generated in a variety of pathologies, including reperfusion injury,[216] proinflammatory cytokine-induced heart failure,[167] doxorubicin-associated cardiotoxicity,[269] allograft rejection,[270] and septic myocardiomyopathy,[188] viral myocardiomyopathy,[188] and atherosclerosis.[271] Based on the discussion in Section 1.1.2, it is like that this potent oxidant activates MMPs in the cardiovascular system during inflammatory stress.

In order to investigate whether oxidative stress plays a role in the activation of myocardial MMPs, authentic peroxynitrite was infused into isolated perfused rat hearts and measured MMP activity.[272] Infusion of 80 μM peroxynitrite for 15 min caused the release of MMP-2 into the perfusate, which was rapidly followed by a significant depression in cardiac mechanical function. It was found that both the release of MMP-2 and the loss of function were both blocked with either the peroxynitrite scavenger glutathione or a synthetic MMP inhibitor (PD-166793). These results suggested that the acute cardiac toxicity induced by exogenous peroxynitrite was mediated by MMP-2.

Rajagopalan *et al* investigated the effect of peroxynitrite on cultured human smooth cells.[47] In this model, it was demonstrated that MMP-2 activity is increased in these cells after exposure to free radical generating systems. Moreover, exposure of MMP-2 to authentic peroxynitrite not only nitrated it in a concentration dependent manner, but also endowed it with collagenolytic activity. Thus, this study demonstrated that MMP activity can be regulated by peroxynitrite not only in cardiac cell types, but also in vascular cell types.

Aside from exogenous applications of this oxidants, peroxynitrite can also be generated endogenously following exposure to pro-inflammatory cytokines. In the isolated perfused rat heart, these cytokines produce a rapidly developing acute heart failure caused by the concurrent upregulation of both nitric oxide and superoxide levels.[167] In accordance with the oxidative stress hypothesis, MMP-2 was found to be activated in cytokine treated hearts and this was followed by a significant decline in cardiac function and degradation of TnI.[168] These effects could be ameliorated with the use of either neutralizing MMP-2 antibody or MMP inhibitors.[168] Thus, this investigation demonstrated that a functionally significant activation of MMPs occurs by endogenously produced peroxynitrite.

Interestingly, it has been observed that the inhibitory activity of TIMP-1 can be reduced upon exposure to peroxynitrite *in vitro*.[273] Thus, in conditions of oxidative stress, particularly those caused by peroxynitrite, it could be predicted that TIMP inhibitory activity would decrease while MMP activity would increase.

Based on these investigations which have demonstrated a link between peroxynitrite and MMPs, it could be predicted that other inflammatory states would also demonstrate an increase in MMP activation. This possibility is explored in subsequent chapters of my thesis.

1.4 Hypothesis and objectives

In this thesis, the central objective which links the various studies is a better understanding of MMP activities in a variety of inflammatory cardiovascular states. For the Chapters 2 and 3, the primary experimental model used is an *in vivo* rat model of sublethal endotoxemia. For Chapter 4 and 5 the focus is switched to stunning injury seen following myocardial I/R, Chapter 4 being a study in isolated rat hearts and Chapter 5 being a clinical study in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass. *My overall hypothesis is that MMP activities are enhanced during inflammatory cardiovascular states and contribute to cardiovascular dysfunction.*

1. Endotoxemia induced cardiovascular dysfunction is partially mediated through MMP activity. (Chapters 2 to 3)

To date, little work has been done examining the role of MMPs in endotoxemia and its clinical correlate, septic shock. My first objective will be to characterize the changes in MMP-2 and -9 activities in an animal model of endotoxemia. Activities will be measured in the plasma (as an indicator of systemic proteolysis), heart, and aorta. Previously, proinflammatory cytokines and peroxynitrite production have been found to be significantly increased in each of these tissues during endotoxemia and have also been shown to contribute to the impairment of cardiovascular function.[106,274-276] Both of these stimuli have also potently regulate the activity of MMPs.[4,18,47,49] Thus, I hypothesize that MMPs are activated during endotoxemia, and are an end effector of cardiovascular dysfunction.

1.1 MMP activity in the plasma, heart, and aorta will be increased during endotoxemia.

1.2 An increase in MMP activity will correlate with the severity of endotoxemia, as measured by changes in mean arterial blood pressure.

1.3 An increase in MMP activity contributes to both cardiac and vascular dysfunction during endotoxemia and proinflammatory stress. Inhibition of MMP activity will ameliorate endotoxemia and cytokine induced cardiac and vascular dysfunction.

2. Matrix metalloproteinase-2 activity in preconditioned hearts following I/R. (Chapter 4)

A number of investigations have now implicated MMPs in cardiac I/R injury. Blocking MMP activity using chemical inhibitors,[58,253,264] neutralizing antibodies,[253] or gene deletion techniques,[255] results in improved cardiac function upon reperfusion. Despite these insights into MMP activity following I/R injury, no investigation to date has examined what happens to MMP activity following ischemic preconditioning. Since preconditioning is an established experimental technique which reduces peroxynitrite formation and also improves cardiac function upon reperfusion,[251,277] I hypothesize that *ischemic preconditioning will decrease the activation and release of MMP-2 following I/R injury.*

3. The involvement of MMPs in clinical I/R injury. (Chapter 5)

In a number of experimental animal models, MMPs have now been implicated in I/R injury. In isolated rat hearts, MMPs were activated and released into the coronary perfusate following I/R injury.[253,264] This activation correlated directly with the ischemic duration, and inversely with the resulting cardiac dysfunction. I/R also resulted in the loss of TIMP-4, an endogenous inhibitor of MMPs, from the heart.[58]

A single study has demonstrated that MMP-2 and –9 activities are increased in the human myocardium following coronary artery bypass grafting with cardiopulmonary bypass (a clinical form of I/R injury).[265] In order to produce a more complete picture of MMPs in clinical I/R injury, MMP activities need to be examined in the immediate minutes following myocardial reperfusion and correlated with ischemic duration and cardiac function following reperfusion. Moreover, since release of MMP activity is pivotal in isolated rat hearts subjected to I/R, the release of myocardial MMPs needs to be examined in the clinical setting. Finally, the effect of I/R on the natural endogenous inhibitors of MMPs needs to be examined. Based on studies of I/R in animal models I hypothesize that:

3.1 MMP-2, –9 activities are increased following myocardial I/R in humans.

3.2 TIMP content is decreased in the myocardium following I/R injury in humans.

3.3 MMP-2 will be activated and released by the myocardium into the plasma following I/R in humans.

3.4 MMP activation will correlate directly with ischemic duration and inversely with cardiac function upon reperfusion.

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CHAPTER 2

ENDOTOXEMIA INDUCED CHANGES IN MATRIX METALLOPROTEINASE-2 AND –9 IN THE HEART AND PLASMA

A portion of this chapter has been submitted for publication. Manoj M. Lalu, Tamás Csont, and Richard Schulz. Endotoxemia induced changes in matrix metalloproteinase-2 and –9 in the heart and plasma. *Critical Care Medicine.*

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2.1 Abstract

Matrix metalloproteinases (MMPs) are key regulators of matrix degradation, remodeling, and repair in the body. We investigated whether myocardial and plasma MMP activities are altered during endotoxemia. Rats were administered either bacterial lipopolysaccharide (LPS) or vehicle (pyrogen-free water). Groups of LPS administered animals were sacrificed at 0.5, 1, 3, 6, 12, and 24 h post-injection. Vehicle injected animals were sacrificed at 6 h. Blood pressure was recorded prior to sacrifice. Heart and plasma samples were analyzed by gelatin zymography and immunoblot.

Blood pressure was significantly depressed at 3 to 24 h post LPS injection, however, overt symptoms of endotoxemia and the reduction in blood pressure were most significant 6 - 12 h post LPS. Heart samples from control rats revealed MMP-2 activity but no MMP-9 activity. MMP-2 activity was significantly depressed when overt symptoms of endotoxemia peaked at 6 - 12 h. Plasma MMP-2 activity significantly decreased 3 - 12 h after LPS injection. This loss of activity was associated with a loss of MMP-2 protein. In contrast, plasma MMP-9 activities were rapidly elevated following LPS injection, peaking between 1 - 12 h. MMP-9 activity correlated inversely with blood pressure.

In a separate set of experiments we assessed the contribution of MMPs to cardiac mechanical dysfunction in endotoxemia. Rats were injected (i.p.) with either LPS or vehicle. MMP inhibitors, either Ro 31-9790 (20 mg/kg), doxycycline (4 mg/kg), or vehicle were administered i.p. 30 min after LPS. At 6 h hearts were excised and perfused as working hearts with Krebs-Henseleit buffer at 37 °C. Cardiac work (cardiac output x

peak systolic pressure product) was measured. Perfusate and ventricle samples were analyzed by gelatin zymography.

Cardiac work was significantly depressed in hearts from LPS-treated rats compared to control rats (control: 55± 4, LPS: 26 ± 6 mmHg*mL*min^{-1}). LPS also caused a loss of 72 kDa MMP-2 activity in the ventricles and the perfusate. Although MMP-9 activity was not detected in the ventricles, LPS resulted in an increase in perfusate 92 kDa MMP-9 activity. The MMP inhibitors significantly improved cardiac function of hearts from LPS-treated rats (Ro 31-9790: 38 ± 3, doxycycline: 51 ± 3 mmHg*mL*min^{-1}), had no effect on the loss of MMP-2 activity, and significantly reduced the MMP-9 activity in the perfusate.

These results demonstrate, for the first time, that LPS induces rapid changes in MMP-2 and MMP-9 activities. Moreoever, MMP inhibitors can significantly preserve cardiac mechanical function during endotoxemia.

2.2 Introduction

Septic shock is a potentially fatal condition in which an unencumbered bacterial infection causes profound vasodilation and cardiac dysfunction (septic cardiomyopathy). This dysfunction arises as a result of an increased production of proinflammatory cytokines and a number of biologically significant circulating factors. These include, but are not limited to, prostaglandins, macrophage migration inhibiting factor,[1] superoxide, and nitric oxide (NO).[2] These last two factors may combine to form the highly toxic oxidant, peroxynitrite,[3] evidence for which has been observed in the heart [4] and lungs [5] of septic patients, and in the plasma of endotoxemic rats.[6]

Despite these insights into the role of NO and peroxynitrite in septic shock, morbidity and mortality related to this pathology remains high and the administration of NO inhibitors in human patients has not been proven as an effective therapy.[7] Thus, in order to advance our understanding of this disease, new factors and potential targets for therapeutics need to be sought out. We speculate that matrix metalloproteinases (MMPs) may be potential mediators of sepsis-related pathologies.

MMPs are a large family of zinc-dependent endopeptidases that are involved in a number of long-term physiological (e.g. embryogenesis, nerve growth) and pathological processes (e.g. atherosclerosis, cancer cell invasion).[8] Recent evidence has suggested that MMPs contribute to cardiac pathologies. Specifically, MMP-2 and MMP-9 are of particular importance in these conditions, as they are present in the plasma[9] and are broadly distributed throughout cardiac tissue.[10] MMP-2 and –9 activities are increased in the human left ventricular myocardium during heart failure[11] and the administration of MMP inhibitors attenuates ventricular remodeling during experimental heart failure.[12] As well, circulating plasma levels of MMP-9 are significantly increased in heart failure patients[9] and are a novel predictor of adverse cardiovascular events in patients with coronary artery disease.[13] Finally, we have demonstrated that MMP-2 is activated during ischemia-reperfusion injury and contributes to the subsequent cardiac dysfunction through the cleavage of troponin I.[14,15]

Interestingly, peroxynitrite has been shown to activate MMPs *in vitro*[16,17] and *ex vivo* in isolated perfused hearts.[18] Peroxynitrite activates the zymogen form of MMPs (proMMP) without cleavage of the propeptide domain. Thus, since there is increased oxidative stress in the form of peroxynitrite in the heart[19] and other organs during sepsis, we hypothesized that MMP activity would also be increased in this pathology. Previous studies have demonstrated that the mRNAs for several MMPs are upregulated in the liver, spleen, and kidney after bacterial lipopolysaccharide (LPS) administration,[20,21] and that selective deletion of the MMP-9 gene protects against LPS induced mortality.[22] Another investigation demonstrated that broad spectrum MMP inhibition significantly improved LPS-induced hypotension in pigs.[23] To date, however, no study has characterized the specific changes in MMP activities during sepsis and correlated these activities to cardiovascular function. In order to investigate these potential changes we used an established model of acute endotoxemia in rats and measured MMP-2 and –9 activities in the plasma and myocardium at various time points. In order to examine the potential role of MMPs in endotoxemic cardiomyopathy we coadministered MMP inhibitors in a separate set of experiments. At the height of endotoxemic symptoms the hearts were excised and perfused *ex vivo* in order to assess cardiac mechanical function and MMP activities in both the ventricles and the perfusate.

2.3 Materials and Methods

This investigation conforms to the *Guide to the Care and Use of Laboratory Animals* published by the Canadian Council on Animal Care (revised 1993).

2.3.1 Rat model of endotoxemia

Male Sprague-Dawley rats (250-300 g) were given either a bolus i.p. injection of a non-lethal dose of lipopolysaccharide (LPS; *Salmonella typhosa* 0901, Difco, 4 mg/kg) or pyrogen-free water (control). Animals were then sacrificed by sodium pentobarbital overdose (100 mg/kg, i.p.) at 0.5, 1, 3, 6, 12, or 24 h. A previous investigation revealed that myocardial NO production is at its highest 6 h after LPS treatment.[24] Control rats were sacrificed at 6 h.

A separate set of rats (3, 6, 12, or 24 h after LPS injection and 6 h control) were anesthetized using pentobarbital (50 mg/kg, i.p.). Rats were placed on a warm heating pad under a lamp in order to maintain body temperature. When adequately anesthetized the femoral artery was exposed and cannulated using saline filled polyethylene tubing which was connected to a Gould P21 pressure transducer. Blood pressure was then measured and recorded using AcqKnowledge 3.1 software.

In both sets of rats a blood sample was drawn from the chest cavity immediately upon sacrifice. The plasma fraction was obtained following centrifugation (6500 g for 5 min at 4 °C) and stored at –20 °C for later determination of plasma nitrite and nitrate (NO_x^-) levels and MMP activities. Hearts were rapidly excised and perfused aerobically for 5 min with oxygenated Krebs buffer at 37 °C by the Langendorff method to rinse them free of blood. Heart ventricles were rapidly frozen in liquid nitrogen and stored at –80°C for later processing.

2.3.2 Administration of MMP inhibitors and isolated heart perfusion

A separate group of rats were given either a bolus intraperitoneal injection of a non-lethal dose of LPS or pyrogen-free water (control, n = 7). 0.5 h later, rats were administered i.p. with the MMP inhibitors Roche 31-9790 (20 mg/kg, n = 10), or doxycycline (4 mg/kg, n = 6), or a volume matched amount of their respective vehicles (polyethylene glycol, n = 6, or pyrogen-free water, n = 10).

6 h post LPS injection animals were sacrificed by sodium pentobarbital overdose (100 mg/kg, i.p.). Hearts were rapidly excised and perfused at 37 °C as working hearts with 110 mL of recirculating Krebs-Henseleit buffer containing 11 mM glucose, 5 mM pyruvate, 100 μU/mL insulin, 1.75 mM Ca^{2+}, 0.5 mM EDTA, and 0.1 % bovine serum albumin.[25,26] This buffer was delivered from the oxygenator (supplied with 95% O_2/5% CO_2) into the left atrium at a preload hydrostatic pressure equivalent to 9.5 mmHg. The perfusate was then ejected by the heart into a compliance chamber (containing 1 mL of air) and into the aortic outflow line. The hydrostatic afterload pressure was set at 70 mmHg.

Heart rate and peak systolic pressure were measured by a Gould P21 pressure transducer in the aortic flow line. Aortic flow and cardiac output were monitored using Transonic flow probes in the afterload and preload lines respectively. Cardiac work, the product of cardiac output (mL*min^{-1}) and peak systolic pressure (mmHg), was noted after 20 min of equilibration in working mode. At this time, 2 mL of perfusate was collected and stored for future processing. The ventricles were snap frozen in liquid nitrogen and stored at –80 °C for later processing.

2.3.2 Determination of plasma NO_x^- levels

Plasma was diluted 1:1 with deionized water and then deproteinized by centrifugal ultrafiltration (Ultrafree-MC microcentrifuge tubes UFC3, Millipore). Ultrafiltrates were analyzed for total nitrate and nitrite content according to the method of Green *et al.*[27] Briefly, all nitrate was first reduced to nitrite on-line by passing the sample through an HPLC column packed with copper coated cadmium. This nitrite was then reacted on-line with Greiss reagent to produce an 'azo' compound which was detected at 546 nm by means of a visible light detector. The concentrations of nitrite in each sample were quantitated by comparison to a standard curve generated using dilutions of sodium nitrite.

2.3.3 Preparation of ventricular homogenates

Frozen ventricular tissue was crushed by a mortar and pestle which was cooled to the temperature of liquid nitrogen. The resulting powder was diluted 1:4 w/v in 50 mM Tris-HCl (pH 7.4) buffer containing 3.1 mM sucrose, 1 mM dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin and 0.1% Triton X-100. This solution was then homogenized with an Ultra-Turrex disperser using four strokes of 4 s duration. The homogenate was centrifuged at 10 000 g for 5 min at 4 °C and the supernatant was kept on ice for immediate assay of MMP activities.

2.3.4 Determination of protein content

Plasma, ventricular homogenate, and perfusate protein contents were determined by the bicinchoninic acid method (BCA kit, Sigma) using bovine serum albumin as a standard.

2.3.5 Measurement of MMP activity by zymography

Gelatinolytic activities of MMPs were examined as previously described.[28,29] 8% polyacrylamide gels copolymerized with gelatin (2 mg/mL, type A from porcine skin, Sigma) were prepared. Non-heated samples were diluted with water in order to load a constant amount of protein per lane (plasma, 20 μg; ventricular homogenate, 40 μg; perfusate, 15 μg). A standard was loaded into one lane of each gel (supernatant of phorbol ester activated HT-1080 cells, American Type Culture Collection) as an internal standard used to normalize activities between gels. Following 1.5 h of electrophoresis, the gels were washed with 2.5% Triton X-100 for 1 h at room temperature (with three changes of solution) to remove sodium dodecyl sulphate. Gels were then incubated for 38 to 48 h at 37°C in incubation buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3). After incubation the gels were stained with 0.05% Coomassie Brilliant Blue (G-250, Sigma) in a mixture of methanol:acetic acid:water (2.5:1:6.5, v/v) and destained in aqueous 4% methanol:8% acetic acid (v/v). Gelatinolytic activities were detected as transparent bands against the dark blue background. Zymograms were digitally scanned and band intensities were quantified using SigmaGel software (Jandel Corporation) and expressed as a ratio to the internal standard. In order to confirm that quantified gelatinolytic activities were of MMP origin, o-phenanthroline (100 μM) was added to the incubation buffer and the gelatinolytic activities were found to be abolished..

2.3.6 Western blot analysis

Either myocardial homogenate (20 μg protein) or plasma (30 μg protein) was loaded onto 8%-10% acrylamide gels, electrophoresed under reducing conditions, and then electroblotted onto polyvinyllidene difluoride membranes (BioRad). Samples were probed with either a mouse anti-human MMP-2 antibody (1:1000 dilution, MAB3308, Chemicon) or a mouse anti-rat MMP-9 antibody which detected the 92 kDa form of this protein (1:200 dilution, MS8A, Neomarkers). In order to measure plasma proteins containing nitrotyrosine, plasma (60 μg protein) was applied to a nitrocellulose membrane (BioRad) using a dot blot apparatus.[30] This membrane was subsequently probed with a rabbit anti-nitrotyrosine antibody (1:500 dilution, 06-284, UpState

Biotechnologies). All blots were subsequently probed with appropriate horseradish peroxidase conjugated antibodies (either anti-mouse or anti-rabbit, Transduction Laboratories), and visualized using the enhanced horseradish peroxidase-luminol chemiluminesence reaction kit (Amersham Pharmacia Biotech).

2.3.7 Statistical Analysis

Results are expressed as the mean ± standard error of the mean (SEM) for *n* animals. The results were analyzed by using Statistical Package for the Social Sciences. One way analysis of variance followed by Fisher's least significant difference post-hoc test was used to evaluate differences between groups. Differences were considered significant at $p< 0.05$.

2.4 Results

2.4.1 LPS administration reduces blood pressure

LPS injection caused a significant reduction in mean arterial blood pressure between 3 and 24 h (Figure 2.1). Blood pressure reached its lowest level compared to control at 6 h post LPS injection ($p<0.0001$). Accompanying this fall in blood pressure was the onset of overt endotoxemic symptoms in the rats which were most apparent 6-12 h after LPS injection. These included lethargic behavior, piloerection, and porphyrin secretion from the eyes. The majority of these symptoms had subsided by 24 h.

2.4.2 Plasma NO_x^- concentration and nitrated proteins

Plasma proteins containing nitrotyrosine were measured by dot blot as a marker of protein modification by peroxynitrite.[3,30] Nitrated proteins were detectable in control rats and became significantly elevated by 6 h following LPS administration (Fig 2.2). Plasma NO_x^- was measured as a marker of NO production. Control rats had a plasma NO_x^- concentration of 11.1 ± 1.7 μM. Injection of LPS caused a time-dependent increase in plasma NO_x^- which was first significantly elevated from the control values 6 h after LPS injection and peaked at 12 h (Table 2.1). By 24 h plasma NO_x^- concentration began to decline towards control levels, but still remained significantly elevated from control levels.

2.4.3 Plasma MMP-2 activities are decreased during endotoxemia

The pattern of reduced myocardial MMP-2 activities was mirrored in the plasma. Plasma from control animals also possessed strong MMP-2 activities at 72 kDa and 75 kDa (Fig 2.3A). After LPS injection, the activity of both decreased over time, the 72 kDa band activity was significantly depressed from 3 to 12 h (Fig 2.3B). By 24 h, the activity of this band was returning to control levels, but still remained statistically lower than control activity. 75 kDa activity was also depressed at 6 and 12 h following LPS injection. The changes in 72 kDa MMP-2 activity were associated with a significant depression in protein content (Fig 2.3C).

2.4.4 Plasma MMP-9 activities increase during endotoxemia

In control plasma, gelatinolytic activities were detected at 92 kDa (proMMP-9), 135 kDa (a lipocalin associated form of proMMP-9 [31]), and 84 kDa (MMP-9) (Fig 2.4A). 1 h after LPS injection, 92 kDa activity significantly rose above control levels (Fig 2.4B). Following this surge in activity, the 92 kDa activity steadily decreased until it was significantly depressed at 12 h and then returned to baseline at 24 h after LPS injection.

The significant depression in 92 kDa MMP-9 activity was associated with a significant decrease in 92 kDa MMP-9 protein at 12 h (Fig 2.4C). The delayed decline in 92 kDa activity was accompanied by increased 84 kDa band activity which was statistically significant at 6 and 12 h. The 135 kDa activity significantly increased 3 h after LPS injection, and plateaued at this elevated level even after 24 h. Plasma 135 kDa and 84 kDa MMP-9 activity at all time points in both control and LPS treated animals showed a significant inverse correlation with mean arterial blood pressure in the same animal (Fig 2.5). However, there were no other correlations between other MMP activities and blood pressure.

2.4.5 MMP inhibitors ameliorate endotoxemia induced cardiac dysfunction

Hearts isolated from 6 h LPS treated animals exhibited significantly depressed aortic flow, peak systolic pressure, and cardiac output compared to hearts taken from control animals ($p<0.05$ for all measurements vs. control, data not shown). As a result, cardiac mechanical function was significantly decreased in hearts taken from LPS-treated animals (Figure 2.6).

The administration of MMP inhibitors (either doxycycline or Roche 31-9790) after LPS administration significantly improved aortic flow, peak systolic pressure, and cardiac output compared to their respective LPS + drug vehicle treated groups ($p<0.05$, data not shown). This resulted in significantly improved cardiac mechanical function (Figure 2.6).

2.4.6 Ventricular and perfusate MMP-2 activities are decreased during endotoxemia

Zymographic analysis of control ventricular tissue (Fig 2.7A) revealed robust 72 kDa and 75 kDa MMP-2 activities. The 72 kDa band corresponded to proMMP-2, and the 75 kDa band corresponded to a rodent-specific glycosylated form of proMMP-2 (Chris Overall, University of British Columbia, personal communication). Following LPS injection, the activity of both 72 and 75 kDa bands decreased and this became statistically significant at 6 and 12 h (Fig 2.7B). By 24 h the activities returned to control levels. The changes in ventricular 72 kDa MMP-2 activity were associated with a significant decrease in MMP-2 protein content at 6 h after LPS administration (Fig 2.7C).

MMP inhibitors had no effect on the LPS induced decrease in 72 kDa MMP activity at 6 h post-LPS administration (Fig 2.8). Perfusate samples from these isolated perfused hearts revealed MMP-2 gelatinolytic bands at 72 kDa and 75 kDa. The pattern of reduced myocardial 72 kDa MMP-2 activity was mirrored in the perfusate of hearts taken from LPS treated animals (Figure 2.9). The administration of MMP inhibitors had no effect on the LPS induced decrease in 72 kDa MMP-2 activity in the perfusate.

2.4.7 MMP inhibitors attenuate the LPS induced increase in perfusate MMP-9 activity

As previously described, MMP-9 activity was not detectable within the normal myocardium,[29] nor was there any evidence for it after endotoxin treatment (data not shown). In contrast, perfusate samples taken from control animals revealed a 92 kDa gelatinolytic activity in zymography. This band was identified as 92 kDa MMP-9 by comparison to the HT 1080 standard. LPS administration significantly increased this activity in the perfusate (Figure 3.3B). The administration of either doxycycline or Roche 31-9790 significantly decreased perfusate 92 kDa MMP-9 activity compared to their respective LPS + vehicle treated groups.

2.5 Discussion

MMPs play an important role regulating the cardiovascular system in pathological states. Since endotoxemia is associated with severe cardiovascular dysfunction, we hypothesized that MMP activity is altered in this pathology. This study quantified, for the first time, the changes in myocardial and plasma MMP-2 and -9 activities during endotoxemia. Moreover, the plasma activities of two forms of MMP-9 (135 kDa and 84 kDa), but not any other MMP, were demonstrated to correlate inversely with blood pressure. We also demonstrated that administration of MMP inhibitors *in vivo* ameliorates LPS induced cardiac dysfunction

Plasma nitrated proteins and NO_x^- concentrations were measured in order to confirm that our animal model of endotoxemia demonstrated an inflammatory response. The increase in nitrated proteins is indicative of an increase in peroxynitrite production during endotoxemia.[6] We have previously demonstrated that 6 h after LPS injection that both reactants of peroxynitrite biosynthesis, nitric oxide and superoxide, as well as nitrotyrosine are increased in the myocardium of rats and this is associated with severe cardiac dysfunction.[19] The increase in plasma levels of stable NO metabolites corroborates the results of a previous investigation using LPS injected rats.[24] This also suggests that our model of endotoxemia emulates the increase in NO production seen in septic patients.[32]

The decrease in ventricular MMP-2 activity within the tissue was unexpected based on our initial hypothesis. Moreover, cell culture studies with LPS stimulation suggest that MMP-2 would be activated.[33,34] However, our previous investigation into the role of MMP-2 in hearts subjected to ischemia-reperfusion injury, another form of oxidative stress shown to be mediated by peroxynitrite,[35] revealed that MMP-2 activity in the heart tissue decreases as the heart actively secretes this enzyme when stressed.[29] Thus, we initially suspected that MMP-2 activity in the plasma may increase as the heart and other organs secrete this enzyme during endotoxemic stress.

However, analysis of MMP-2 activity in the plasma revealed an overall decrease during endotoxemia. Again, in light of our initial hypothesis, these results were surprising. The release of MMP from the heart (and other organs) may still occur, however, this may not be detected in the plasma as it could be either rapidly inactivated or removed from the circulation as a means to prevent further proteolytic stress. Interestingly, the loss of MMP-2 activity was closely related to changes in MMP-2 protein content as measured by immunoblot. Thus, the overall decrease in MMP-2 activity is likely due to decreased levels of MMP-2 protein. This decrease in myocardial MMP-2 activity may in fact be a cardioprotective mechanism. With less MMP-2 activity, the heart would be subjected to less proteolytic stress from this enzyme which has been implicated in a number of cardiac pathologies. MMP-2 activation and release may have already occurred *in vivo* prior to the cardiac dysfunction noted *ex vivo* at 6 h post LPS injection. Indeed, in isolated rat hearts perfused with proinflammatory cytokines we have noted that MMP-2 is activated and released into the perfusate within the first 30 min of exposure[36] and that MMP-2 mRNA expression is subsequently downregulated (unpublished observations, Schulz lab). Regardless of the exact mechanism of loss of MMP-2 activity, the demonstration that MMP-2 activity is regulated acutely *in vivo* is novel, and opposes the notion that MMP-2 is merely a ubiquitous 'house-keeping' protein.[37]

The increase in MMP-9 activity in the plasma and cardiac perfusate conforms to the idea that MMP-9 is a rapidly inducible enzyme under the pro-inflammatory cytokine conditions of endotoxemia. The time-course profile of 92 kDa MMP-9 activity in our study mirrors that seen in isolated murine peritoneal macrophages stimulated with LPS.[33] This time-course profile suggests that 92 kDa MMP-9 is released rapidly into the circulation following LPS injection. The increase in 135 kDa MMP-9 implicates activated neutrophils as a potential source of this increased MMP-9 activity.[31] The delayed increase in the 84 kDa MMP-9 activity suggests that the 92 kDa MMP-9 is converted to the 84 kDa form in this model of endotoxemia as the condition of the animal deteriorates.

In hearts taken from LPS treated rats 92 kDa MMP-9 activity could be detected in the cardiac perfusate. However, one cannot accurately state that the 92 kDa MMP-9 we detected in both plasma and perfusate is inactive, as it has been shown that this MMP-9 is activated by peroxynitrite through a unique glutathiolation reaction.[16] As well, peroxynitrite infusion *ex vivo* into isolated perfused rat hearts can activate MMPs without proteolytic cleavage.[18] Such an activation could explain the discordance between plasma MMP-9 activity and protein content at 3 h post LPS injection, a time when MMP-9 activity is significantly increased but protein expression is not. Thus, the increased release and activity of 92 kDa MMP-9 in our model of endotoxemia may represent a peroxynitrite induced release of MMP-9.

The inhibitors used in this study were structurally unrelated and inhibit MMPs through different mechanisms. Doxycylcine is a tetracycline antibiotic which inhibits active MMPs through direct chelation of the catalytic zinc site,[38] while Roche 31-9790 is a hydroxamate inhibitor which acts as pseudosubstrate.[39] The specificity of these drugs was maintained by administering *in vivo* doses lower than any previous report.[40-43] However, since doxycycline and Roche 31-9790 are both broad spectrum inhibitors of MMPs, we cannot exclude the possibility that the beneficial effects noted in this study were related to inhibition of other MMPs which were not analyzed by gelatin zymography.

Previous investigations have demonstrated that septic patients have increased circulating levels of MMP-9 protein,[44] that human volunteers infused with LPS have increased plasma activity of MMP-9,[45] and that the deletion of the MMP-9 gene has been shown to protect mice from endotoxemia.[22] In our study we have demonstrated for the first time that activity of 135 kDa and 84 kDa MMP-9, but not other MMP activities, correlated inversely with the mean arterial blood pressure of the animal. The rise in MMP-9 activity may contribute to an overall increase in the proteolytic state of septic patients[46,47] and may be a marker of the severity of sepsis.[44] The exact proteolytic targets of MMP-9 during endotoxemia remain to be discovered.

In summary, this investigation revealed a number of novel and exciting results. The administration of LPS caused a significant decline in MMP-2 activity in both myocardium and plasma. During endotoxemia there was also a rapid and significant increase in MMP-9 activity in the plasma. The activities of 135 kDa and 84 kDa MMP-9 correlated negatively with blood pressure at all time points. This increase in activity likely contributes to an overall increase in the proteolytic state of these animals. We have also demonstrated that MMP inhibitors prevent LPS induced cardiac dysfunction and the associated increase in perfusate MMP-9 activity. These results offer further evidence that MMP activity can be regulated not only in chronic conditions, but also acutely *in vivo.* Moreover, although MMP inhibition has been touted as a potential therapy in preventing cardiac remodeling, our results suggest that MMP inhibition may also prevent acute endotoxemic cardiac dysfunction.

Table 2.1 Plasma NO_x^- concentrations following lipopolysaccharide (LPS) administration

Group		**NO_x^- concentration (μM)**		
Control		11.1	±	1.7
LPS	0.5 h	11.1	±	2.5
	1 h	11.1	±	2.0
	3 h	22.5	±	3.1
	6 h	217.8	±	14.9 *
	12 h	580.6	±	88.6 *
	24 h	137.9	±	65.4 *

*$p<0.05$ versus Control

(Data compiled with help from K. Strynadka)

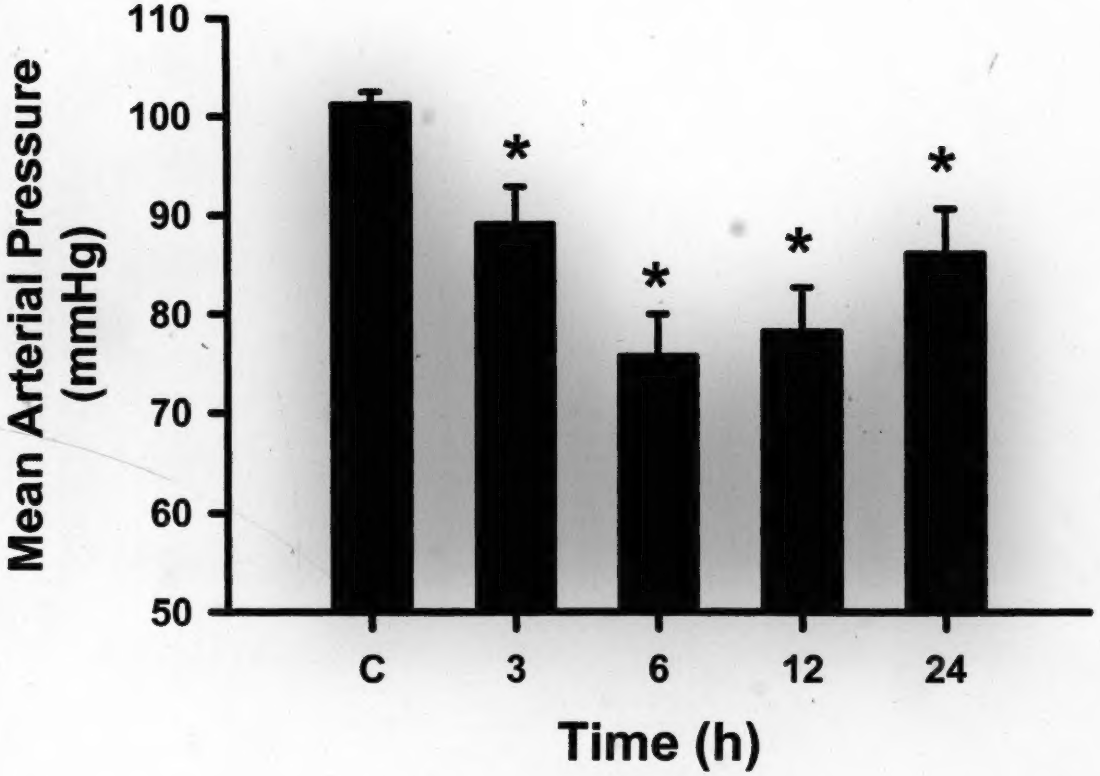

Figure 2.1 Time course of changes in mean arterial blood pressure following lipopolysaccharide injection. C represents control values 6 h after injection with pyrogen free water. n = 4-7 animals per group, $^*p < 0.05$ versus control animals, one way ANOVA. *(Blood pressure measurements done with help from T. Csont)*

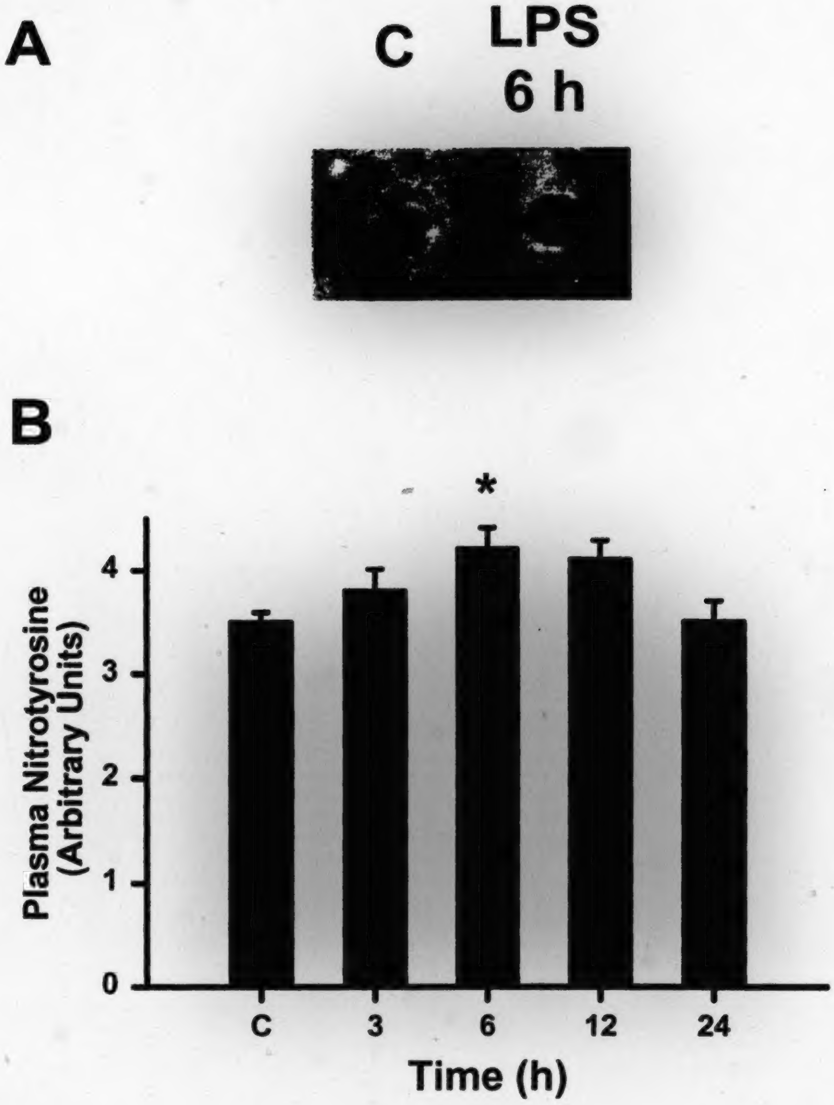

Figure 2.2 Time course of changes in plasma proteins containing nitrotyrosine following lipopolysaccharide injection. (A) A representative dot blot of proteins containing nitrotyrosine. C represents control animals 6 h after injection with pyrogen free water. n = 4-7 animals per group, *$p < 0.05$ versus control animals, one-way ANOVA.

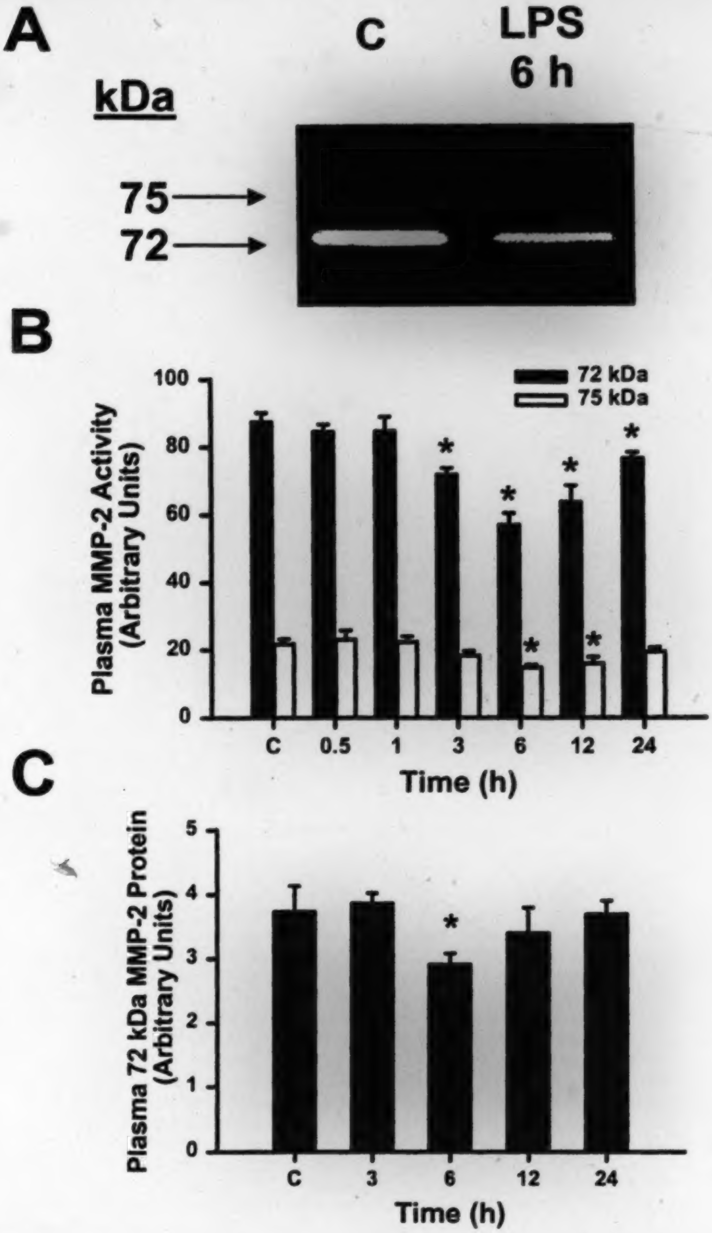

Figure 2.3 (A) A representative zymogram of matrix metalloproteinase-2 gelatinolytic activities in the plasma. Plasma from one control animal (left lane) and one lipopolysaccharide treated animal (6 h after injection; right lane) have 75 kDa and 72 kDa activities. Gel incubation time: 38 h. (B) Time course of changes in plasma matrix metalloproteinase-2 activities following lipopolysaccharide injection. (C) Time course of changes in plasma 72 kDa matrix metalloproteinase-2 protein content following lipopolysaccharide injection. n = 4-7 animals per group, * $p < 0.05$ versus control animals, one-way ANOVA.

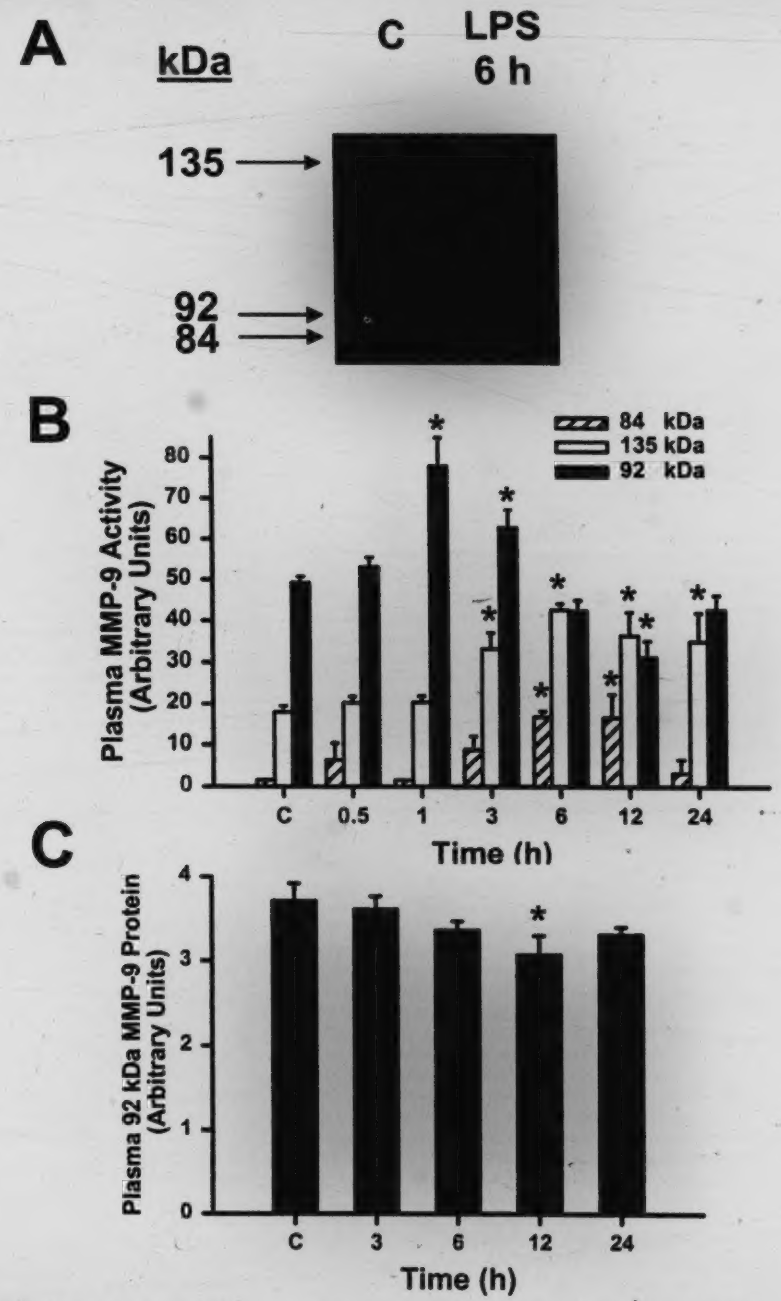

Figure 2.4 (A) A representative zymogram of matrix metalloproteinase-9 activities in the plasma. Plasma from one control animal (left lane) shows 135 kDa and 92 kDa activities. Plasma from one lipopolysaccharide treated animal (6 h after injection; right lane) shows 135 kDa, 92 kDa, and 84 kDa activities. Gel incubation time: 48 h. (B) Time course of changes in plasma matrix metalloproteinase-9 activities following lipopolysaccharide injection. (C) Time course of changes in plasma 92 kDa matrix metalloproteinase-9 protein content following lipopolysaccharide injection. n = 4-7 animals per group, * $p < 0.05$ versus control animals.

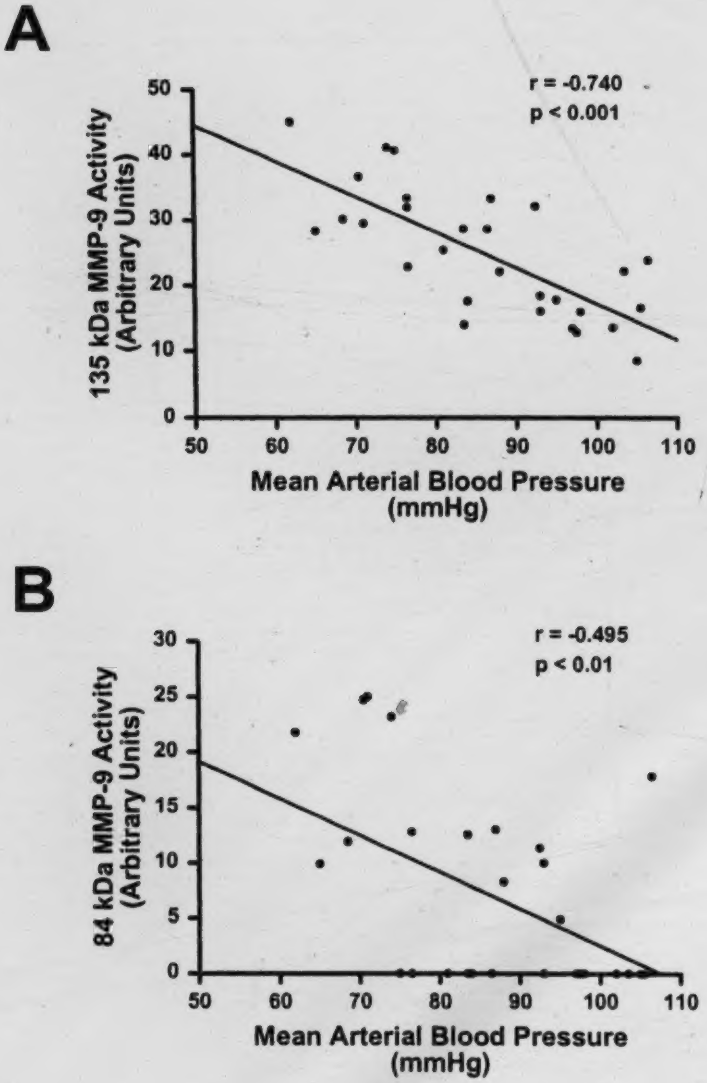

Figure 2.5 Mean arterial blood pressure measured at any time point shows an inverse correlation with (A) 135 kDa MMP-9 activity and (B) 84 kDa MMP-9 activity in both control and LPS treated animals.

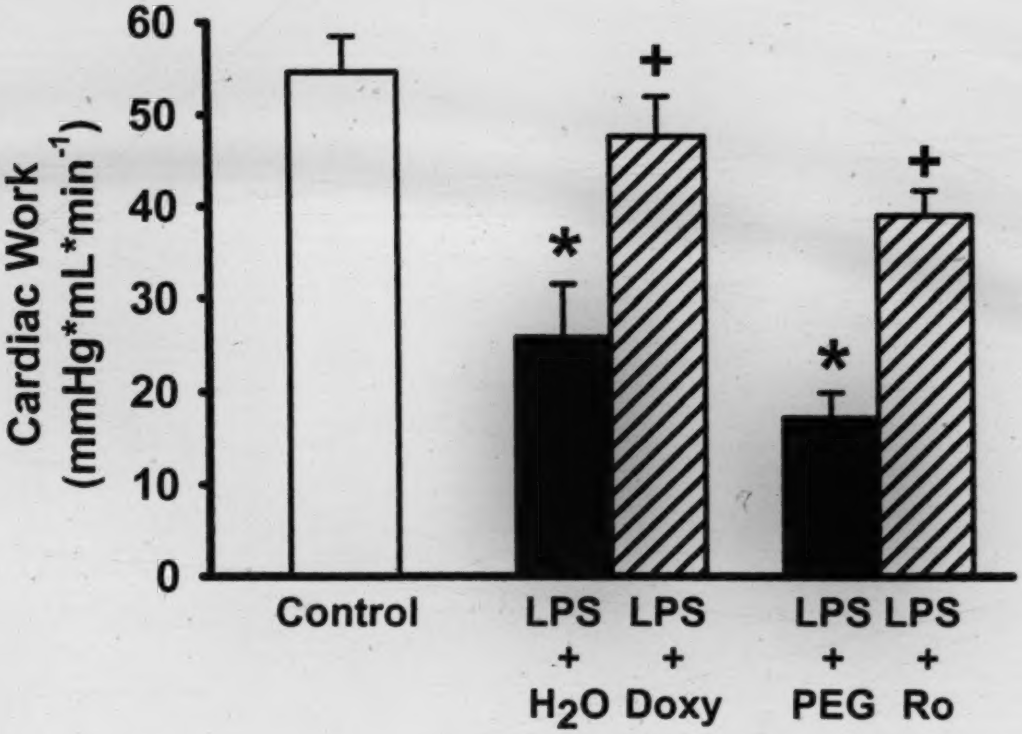

Figure 2.6 Cardiac work in hearts isolated from control or LPS treated rats with or without MMP inhibitors. Cardiac mechanical function was measured following 20 min of equilibration in working mode. LPS = lipopolysaccharide, Doxy = doxycycline, PEG = polyethylene glycol, and Ro = Roche 31-9790. n = 7 – 10 animals per group. * = $p < 0.05$ vs. control, + = $p < 0.05$ vs. respective LPS + vehicle treated group, one-way ANOVA. *(Heart perfusions done with help from CQ Gao.)*

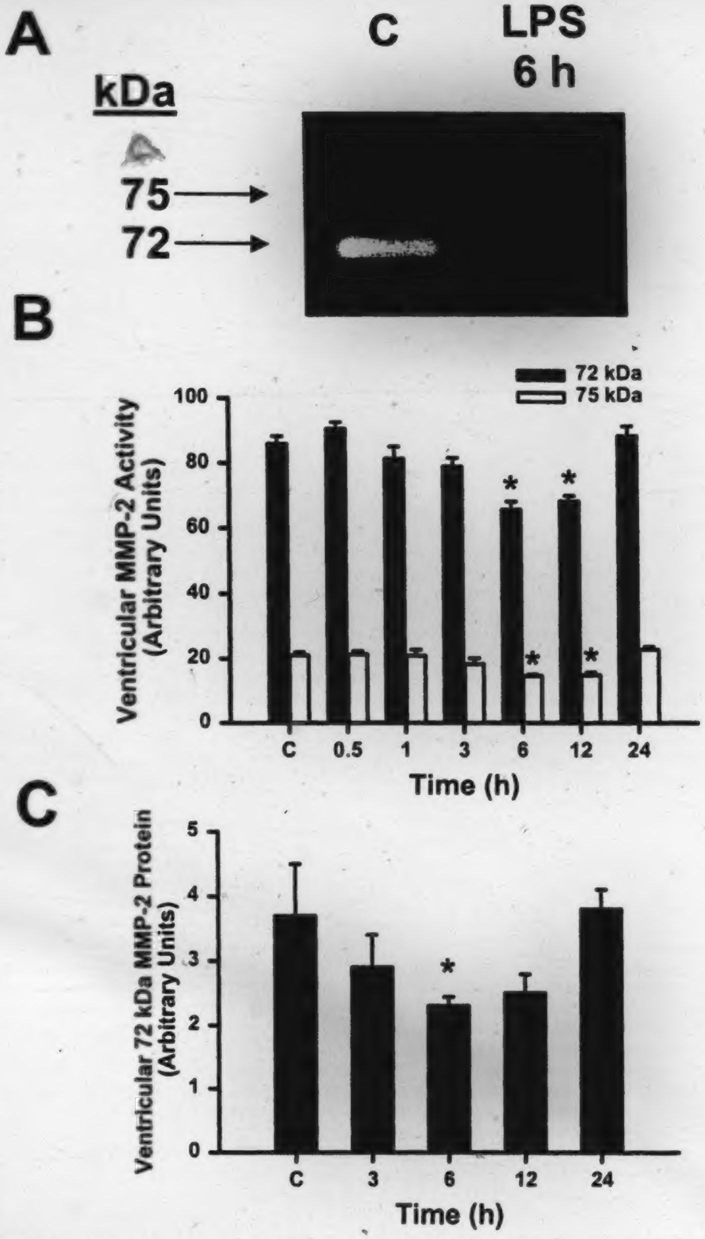

Figure 2.7 (A) A representative zymogram of ventricular gelatinolytic activities. Ventricular homogenates from one control animal (left lane) and one lipopolysaccharide treated animal (6 h after injection; right lane) have 75 kDa and 72 kDa activities. No matrix metalloproteinase-9 gelatinolytic bands were detected. Gel incubation time: 38 h. (B) Time course of changes in ventricular matrix metalloproteinase-2 activities following lipopolysaccharide injection. (C) Time course of changes in ventricular 72 kDa matrix metalloproteinase-2 protein content following lipopolysaccharide injection. C represents control values 6 h after injection with pyrogen-free water n = 4-7 animals per group, * $p < 0.05$ versus control animals, one-way ANOVA.

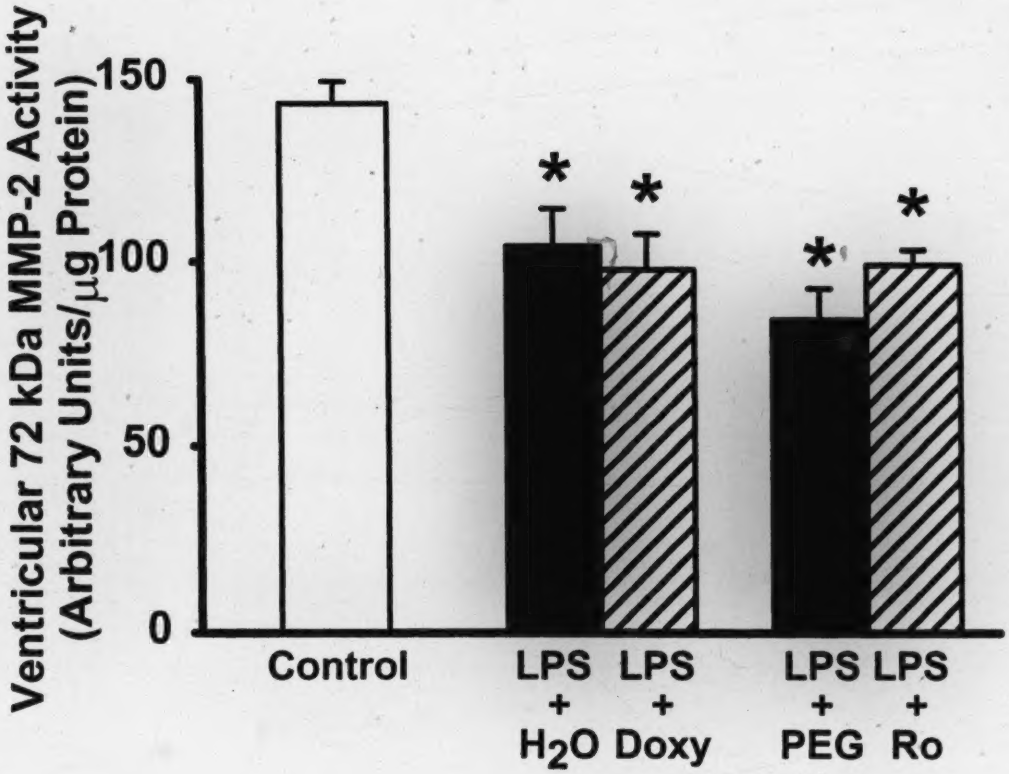

Figure 2.8 Ventricular 72 kDa MMP activity in control and experimental groups 6 h post injection. 72 kDa MMP activity, the primary gelatinolytic activity in rat myocardium, was measured at the end of perfusion by gelatin zymography and quantified by densitometric analysis. n = 7 – 10 animals per group, * = $p < 0.05$ vs. control, one-way ANOVA.

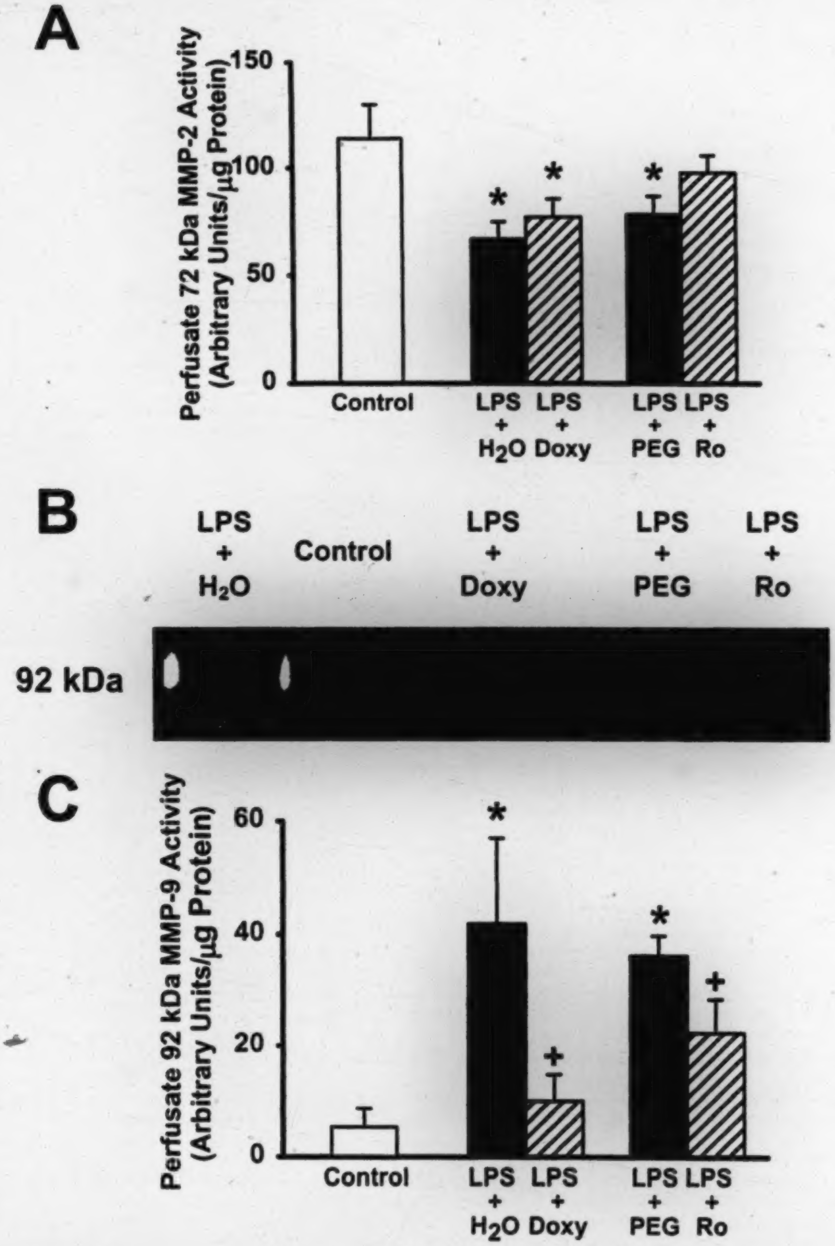

Figure 2.9 Perfusate MMP activities in control and experimental groups 6 h post injection. (A) 72 kDa MMP-2 and (B-C) 92 kDa MMP-9 activities were measured by gelatin zymography and analyzed by densitometric analysis in perfusate samples collected after 20 min of stabilization in working mode. (B) Representative 92 kDa activities from different groups. n = 7 – 10 animals per group, * = $p < 0.05$ vs. control, + = $p < 0.05$ vs. respective LPS + vehicle treated group, one-way ANOVA.

2.6 References

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CHAPTER 3

MATRIX METALLOPROTEINASES CONTRIBUTE TO ACUTE INFLAMMATORY VASCULAR DYSFUNCTION

A version of this chapter has been submitted for publication. Manoj M. Lalu, Rezwan Chowdhury, and Richard Schulz. Matrix metalloproteinases contribute to acute inflammatory vascular dysfunction. *Arteriosclerosis, Thrombosis, and Vascular Biology.*

3.1 Abstract

An enhanced proteolytic state caused by an imbalance between matrix metalloproteinases (MMPs) and their natural inhibitors (tissue inhibitors of MMPs, TIMPs) has been implicated in numerous cardiovascular pathologies. We hypothesized that an imbalance between MMPs and TIMPs contributes to acute impairment in vascular contractile function under severe inflammatory conditions, such as that seen in endotoxemia. Aortic rings from normal rats were mounted in an organ bath, precontracted with phenylephrine (750 nM), and monitored for LPS-mediated spontaneous loss of contractile tone. After 6 h, control aortas maintained 58 ± 4% of their tone, while aortas treated *in vitro* with MMP inhibitors, either doxycycline (30 μM) or GM6001 (10 μM), maintained 101 ± 3% and 99 ± 5% of their tone, respectively ($p<0.05$). In order to determine whether MMP inhibition could protect against proinflammatory cytokine induced contractile dysfunction, aortic rings were incubated under cell culture conditions for 6 h with interleukin-1β (10ng/mL) and in the presence or absence of GM6001 (10 μM). Rings were then mounted in an organ bath and contracted with phenylephrine. IL-1β treated rings had a significantly reduced contractile response to phenylephrine compared to control rings, and treatment with GM6001 significantly improved this response. In order to assess whether MMPs and TIMPs are altered *in vivo* in an inflammatory state, rats were injected with either lipopolysaccharide (LPS, 4 mg/kg) or pyrogen-free water vehicle (control) and sacrificed 6 h later. Aortas were excised, homogenized, and analyzed using net gelatinase and collagenase activity assays, gelatin zymography (for MMP-2 and MMP-9 activities) and immunoblot (for MMP-2, MMP-9, TIMP-1, TIMP-2, and TIMP-4 protein content). Net gelatinolytic

activity was increased in aortas from LPS treated rats compared to control rats, while collagenolytic activity remained unchanged. 72 kDa MMP-2 and 92 kDa MMP-9 activities were significantly increased, relative to their protein content, in LPS aortas. TIMP-4 protein content was significantly reduced and TIMP-1 was significantly increased in LPS aortas, while TIMP-2 remained unchanged. This is the first demonstration that MMP inhibition ameliorates both LPS and cytokine induced vascular dysfunction. Moreover, acute changes occur in vascular MMPs and TIMPs under inflammatory conditions.

3.2 Introduction

Matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases which are key regulators of the extracellular matrix. The gelatinases, MMP-2 and MMP-9, contribute to a wide variety of chronic cardiovascular pathologies including heart failure, atherosclerosis, and abdominal aneurysms (reviewed in References [1,2]). Regulation of their main endogenous inhibitors, tissue inhibitors of MMPs (TIMPs) may also play a role in these chronic pathologies.

Recently, MMP-2 has been implicated in a number of acute processes, such as myocardial ischemia-reperfusion injury,[3-5] regulation of normal vascular tone,[6,7], platelet aggregation,[8] and modulation of the inflammatory response.[9] Other acute conditions which MMPs may be involved in are endotoxemia and its closest clinical correlate, septic shock.

Septic shock is a potentially fatal condition in which a systemic bacterial infection produces an unencumbered inflammatory response. This inflammatory response then produces cardiovascular dysfunction that is characterized by vasodilation and cardiac dysfunction. It is the chief cause of death and disability in intensive care units.[10] A similar response and cardiovascular dysfunction can be provoked by administering endotoxin (lipopolysaccharide, LPS) to animals or human volunteers.[11,12]

In both sepsis and endotoxemia the initial inflammatory response is largely mediated by a 'proinflammatory cytokine storm' (including, interleukin-1β,[13] tumor necrosis factor-α[14,15]) which increases the production of a number of downstream effectors. One effector is peroxynitrite,[16] the toxic reaction product of excess nitric oxide[17] and superoxide anion[18] produced during severe acute inflammation. Peroxynitrite reacts with a number of different proteins[19] and lipids,[20] to cause structural damage, enzyme dysfunction, and eventually cell death. Interestingly, both proinflammatory cytokines[21,22] and peroxynitrite[23-26] have been demonstrated to increase MMP activity and decrease TIMP activity *in vitro.* Thus it was speculated that an imbalance between MMPs and TIMPs occurs during acute inflammatory stress.

To date, few investigations have examined the role of MMPs in septic shock and endotoxemia. In experiments using endothelial cells, neutrophils, and macrophages, LPS was found to increase MMP-2 and MMP-9 activities.[27-29] In both animal and human models of endotoxemia circulating MMP-9 activity is increased,[29-32] while in true sepsis in both animal models and in humans circulating MMP-2 and MMP-9 are elevated.[31,33,34] Despite these insights, no study has investigated the role of MMPs in the vascular dysfunction that arises from severe, acute inflammatory stress.

In order to address this issue, we employed three previously established models of vascular inflammatory stress. First, the effect of MMP inhibition on both LPS and cytokine mediated vascular dysfunction was investigated. Next, the alterations in MMPs and TIMPs were investigated using a well-established rat model of endotoxemia.

3.3 Materials and Methods

This investigation conforms to the *Guide to the Care and Use of Laboratory Animals* published by the Canadian Council on Animal Care (revised 1993).

3.3.1 LPS mediated spontaneous loss tone in aortic rings

Untreated male Sprague-Dawley rats were sacrificed by sodium pentobarbital overdose (100 mg/kg, i.p.). Aortas were rapidly excised and connective tissue was trimmed away in gassed (95% O_2-5% CO_2) Krebs buffer (118 mM NaCl, 4.75 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4 \cdot 7\ H_2O$, 2.5 mM $CaCl_2 \cdot 2\ H_2O$, 11.1 mM D-glucose, 25 mM $NaHCO_3$) at room temperature. Rings (5 mm in length) were mounted in organ baths filled with Krebs buffer at 37 °C which was oxygenated with 95% O_2-5% CO_2. Isometric tension was recorded using force transducers (Grass FT03), and recorded using AcqKnowledge 3.1 Software. A tension of 1 g was applied and the rings were equilibrated for 60 min with fresh Krebs buffer added at intervals of 20 min. Following equilibration, rings were contracted with phenylephrine (750 nM, Sigma) and at the plateau of contraction acetylcholine bromide (10 μM, Sigma) was added to assess endothelial integrity. Rings that did not relax by at least 80% of the phenylephrine contraction were not used. Rings were then washed and constricted again with phenylephrine (750 nM). At the plateau of contraction one of the following was added: polymyxin B (10 μg/mL, Sigma), doxycycline (10 μM, Sigma), vehicle for doxycycline

(ddH_2O), GM6001 (10 μM, Chemicon or Calbiochem), or vehicle for GM6001 (0.1% ethanol in ddH_2O). Vascular tone was then monitored for another 4.5 h.

3.3.2 IL-1β stimulated vascular dysfunction

Untreated male Sprague-Dawley rats were sacrificed as described above. Aortas were rapidly excised, washed three times in sterile phosphate buffered saline (supplemented with an antibiotic cocktail: 100 μg/mL streptomycin, 100 U/mL penicillin, 5 μg/mL gentamicin, Sigma), and then dissected under a tissue culture hood. Rings were cut (5 mm in length) and then washed three times in phosphate buffered saline before a final wash in Dulbecco's modified Eagle's medium (with 1000 mg/L glucose, pyroxidine HCl, $NaHCO_3$, supplemented with antibiotic cocktail, Sigma). Rings were then placed in fresh cell culture medium with one or more of the following added: IL-1β (10 ng/mL, R & D Systems), GM6001 (10 μM), GM6001 vehicle (100% ethanol). Samples included in each experimental group were taken from distinct animals. Rings were then incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 6 h rings were removed from the cell culture medium and mounted in an organ bath as described above. Following equilibration (60 min) under 1 g of tension, a concentration response curve to phenylephrine was obtained, and endothelial integrity was assessed by addition of 10 μM acetylcholine to the same criteria as described in 4.3.1 above. Rings were then washed and the maximum response to KCl (75 mM) was determined.

3.3.3 Rat model of endotoxemia

Male Sprague-Dawley rats (250-300 g) were given either a bolus i.p. injection of a non-lethal dose of lipopolysaccharide (LPS; *Salmonella typhosa*, Sigma, 4 mg/kg) or pyrogen-free water (control). Rats were then sacrificed by sodium pentobarbital overdose (100 mg/kg, i.p.) at 6 h. Previous investigations have revealed that in this model of endotoxemia, NO production is increased by this time point, and that cardiovascular function is significantly depressed.[32,35,36]

In both sets of rats a blood sample was drawn from the chest cavity immediately upon sacrifice. The plasma fraction was obtained following centrifugation (6500 g for 5 min at 4 °C) and stored at –20 °C for later determination of plasma nitrite and nitrate (NO_x^-) levels. Aortas were rapidly excised and fatty tissue was trimmed away in gassed Krebs buffer. The dissected aortas were blotted and then rapidly frozen in liquid nitrogen and stored at –80°C for later processing.

3.3.4 Determination of plasma NO_x^- levels

Plasma was diluted 1:1 with deionized water and then deproteinized by centrifugal ultrafiltration (Ultrafree-MC microcentrifuge tubes UFC3, Millipore). Ultrafiltrates were analyzed for total nitrate and nitrite content according to the method of Green *et al.*[37] Briefly, all nitrate was first reduced to nitrite on-line by passing the sample through an HPLC column packed with copper coated cadmium. This nitrite was then reacted on-line with Greiss reagent to produce an 'azo' compound which was detected at 546 nm by means of a visible light detector. The concentrations of nitrite in each sample were quantitated by comparison to a standard curve generated using dilutions of sodium nitrite.

3.3.5 Preparation of aorta homogenates

Frozen aorta tissue was crushed by a mortar and pestle which was cooled with dry ice. The resulting powder was diluted 1:4 w/v in 50 mM Tris-HCl (pH 7.4) buffer containing 3.1 mM sucrose, 1 mM dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin and 0.1% Triton X-100. This solution was then homogenized on ice by hand using a microcentrifuge tube pestle . The homogenate was centrifuged at 10 000 g for 5 min at 4 °C and the supernatant was kept on ice for immediate assay of MMP activities.

3.3.6 Determination of protein content

Aorta homogenate protein contents were determined by the bicinchoninic acid method (BCA kit, Sigma) using bovine serum albumin as a standard.

3.3.7 Gelatinase and collagenase assay

In order to measure the activities of collagenases (MMP-1, -8, -13), aortic homogenates (50 μg of protein) were analyzed using an MMP collagenase assay kit (ECM710, Chemicon) according to manufacturers' instructions. The samples, however, were not chemically treated to activate latent collagenase activity. Briefly, biotinylated collagen was digested by collagenase activity in the samples at 37 °C. The biotinylated fragments were then transferred to a biotin binding 96 well plate and detected with a streptavidin-enzyme complex. Addition of a colorimetric substrate produced a colored reaction product which was detectable at 450 nm. Addition of 10 μM GM6001 was found to abolish all collagenase activity.

In order to measure the net activity of gelatinases (primarily MMP-2 and –9) aortic homogenate (100 μg of protein) was analyzed using a gelatinase assay kit (E-12055, Molecular Probes). Non-activated samples were incubated at room temperature in the presence of DQ gelatin fluorescein conjugate. Digestion of this product yields fluorescent peptides which were detectable on a fluorometer (λ_{ex} 495 nm, λ_{em} 515 nm). In order to subtract gelatinase activity not attributable to MMP activity, samples were also loaded in the presence of 100 μM o-phenanthroline.

3.3.8 Measurement of MMP activity by zymography

Gelatinolytic activities of MMPs were examined by gelatin zymography as previously described.[5,38] 8% polyacrylamide gels copolymerized with gelatin (2 mg/mL, type A from porcine skin, Sigma) were prepared. Non-heated samples were diluted with water in order to load a constant amount of protein per lane (20 μg of protein from aortic homogenate). A standard was loaded into one lane of each gel (supernatant of phorbol ester activated HT-1080 cells, American Type Culture Collection) as an internal standard used to normalize activities between gels. Following 1.5 h of electrophoresis, the gels were washed with 2.5% Triton X-100 for 1 h at room temperature (with three changes of solution) to remove sodium dodecyl sulphate. Gels were then incubated for 20 to 30 h at 37°C in incubation buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3). After incubation the gels were stained with 0.05% Coomassie Brilliant Blue (G-250, Sigma) in a mixture of methanol:acetic acid:water (2.5:1:6.5, v/v) and destained in aqueous 4% methanol:8% acetic acid (v/v). Gelatinolytic activities were detected as transparent bands against the dark blue background. Zymograms were digitally scanned and band intensities were quantified using SigmaGel software (Jandel Corporation) and expressed as a ratio to the internal standard. In order to confirm that quantified gelatinolytic activities were of MMP origin, o-phenanthroline (100 μM) was added to the incubation buffer and the gelatinolytic activities were found to be abolished..

3.3.9 Immunoblot analysis

Aorta homogenate (10 – 20 μg protein) was loaded onto 8% or 12% polyacrylamide gels, electrophoresed under reducing conditions, and then electroblotted onto polyvinyllidene difluoride membranes (BioRad). Positive standards and/or molecular weight standards were also loaded into gels in order confirm identity of proteins to be probed. Samples were probed with either a mouse anti-human MMP-2 antibody (1:1000 dilution, MAB3308, Chemicon), a rabbit anti-rat MMP-9 antibody which detected the 92 kDa form of this protein (1:4000 dilution, courtesy of Dr. Mieczyslaw Wozniak, Medical University, Wroclaw, Poland), mouse anti-human TIMP-1 antibody (1:100 dilution, MS-608, NeoMarkers), rabbit anti-human TIMP-2 antibody (1:100 dilution, RB-1489, NeoMarkers), or a rabbit anti-human TIMP-4 antibody (1:5000 dilution, AB19087, Chemicon). All blots were subsequently probed with appropriate horseradish peroxidase conjugated antibodies (either anti-mouse or anti-rabbit, Transduction Laboratories), and visualized using the horseradish peroxidase-luminol chemiluminesence reaction kit (Amersham Pharmacia Biotech).

3.3.10 Statistical Analysis

Results are expressed as the mean ± standard error of the mean (SEM) for *n* animals. The results were analyzed by using Statistical Package for the Social Sciences. Independent samples t-test or repeated measures two-way ANOVA was used as indicated to evaluate differences between groups. Differences were considered significant at $p < 0.05$.

3.4 Results

3.4.1 MMP inhibition ameliorates LPS-mediated loss of vascular tone

In order to assess whether MMP inhibition affects LPS mediated vascular dysfunction, aortas from normal rats were mounted in organ baths, then contracted with phenylephrine, and their tone was monitored for 4.5 h. A spontaneous and significant loss of vascular tone was noted in these contracted vessels (52 ± 4 % of original phenylephrine-induced tone was maintained, Fig 4.1A), which has previously been demonstrated to be due to ambient LPS.[17] Addition of polymyxin B (10 μg/mL), a drug which binds and chelates LPS, following constriction with phenylephrine abolished this loss of tone ($p < 0.05$, data not shown). In order to evaluate the efficacy of MMP inhibition in this model, two pharmacologically distinct MMP inhibitors were tested. The addition of doxycycline (30 μM) completely protected the vessels from the spontaneous loss in vascular tone (101 ± 3% of tone maintained, $p < 0.05$, Fig 4.1A). The addition of GM6001 (10 μM) also significantly protected the vessels from a spontaneous loss of tone (99 ± 5% of tone maintained, $p < 0.05$, Fig 4.1B).

3.4.2 MMP inhibition ameliorates IL-1β mediated vascular dysfunction

A model of cytokine mediated vascular dysfunction was used in order to rule out that MMPs inhibitors protected against LPS mediated dysfunction by inhibiting cytokine processing,. IL-1β (10 ng/mL) was added to aorta rings incubated in cell culture conditions for 6 h. IL-1β treatment significantly decreased the contractile response to cumulative concentrations of phenylephrine relative to control vessels that were incubated without cytokine ($p < 0.05$, Fig 4.2, solid curves). The addition of 10 μM GM6001 to the cell culture medium significantly protected the vessels from cytokine mediated dysfunction ($p < 0.05$, Fig 4.2). The addition of GM6001 had no effect on the contractile response of control vessels, nor did the drug vehicle have any effect on cytokine treated vessels (Fig 4.2). The maximum response to KCl (75 mM) did not differ between groups (data not shown).

3.4.3 LPS administration causes overt signs of endotoxemia

Overt endotoxemic symptoms were apparent in rats 6 h following LPS administration. These included lethargic behavior, piloerection, and porphyrin secretion from the eyes. Plasma NO_x^-, measured as a marker of NO biosynthesis, was significantly elevated at this time point relative to vehicle treated (control) rats (32 ± 4 μM in control rats, 330 ± 37 μM in LPS treated rats, $p<0.05$).

3.4.4 Aortic gelatinolytic activity is increased following LPS administration

6 h after LPS administration net gelatinolytic activity in excised aortas was found to be significantly increased relative to aortas from control rats ($p<0.05$, Figure 4.3A). Since gelatin is susceptible to cleavage by collagenases, collagenolytic activity was also measured. Net collagenolytic activity in the aorta, however, was not increased relative to control rats (Fig 4.3B). Thus, the increase in gelatinolytic activity was most likely dependent on MMP-2 and MMP-9.

3.4.5 LPS administration increases MMP-2 and MMP-9 activity relative to protein content *in vivo*

Zymographic analysis of control aortic tissue (Fig 4.4A) revealed robust 72 kDa gelatinolytic activity, as well as minor 75 kDa and 64 kDa activities. The rank order of gelatinolytic activities was 72 kDa > 75 kDa > 64 kDa. The 72 kDa and 64 kDa bands corresponded to MMP-2 by comparison to the standard, and the 75 kDa band corresponded to a rodent-specific glycosylated form of proMMP-2 (Chris Overall, University of British Columbia, personal communication). The 72 kDa band activity was quantified, and following LPS administration no significant change in activity was noted (Fig 4.4A). In contrast, however, 72 kDa MMP-2 protein content was significantly decreased following LPS administration ($p<0.05$, Fig 4.4A).

In aortas from control rats, gelatinolytic activity of molecular weight higher than 75 kDa (the region for MMP-9) was not detectable. However, when zymographic gels were incubated for a longer period of time, 92 kDa MMP-9 activity could be detected only in LPS treated rats ($p<0.05$, Fig 4.4B). This activity corresponded to MMP-9 activity by comparison to the standard. Interestingly, immunoblot revealed that 92 kDa MMP-9 protein content was not significantly different between control and LPS treated rats (Fig 4.4B).

3.4.6 LPS administration affects TIMP-1 and TIMP-4 protein content

Immunoblot analysis was also performed to assess aortic TIMP-1, -2, and –4 content. TIMP-1 protein was found to be increased almost three fold in LPS treated rats relative to control rats ($p<0.05$, Fig 4.5A). On the other hand, TIMP-4 protein content was significantly decreased in LPS treated rats ($p < 0.05$, Fig 4.5C). TIMP-2 protein content was not significantly different between the two groups (Fig 4.5B).

3.5 Discussion

We studied the effect of MMP inhibition on LPS and proinflammatory cytokine mediated vascular dysfunction, as well as the regulation of vascular MMP and TIMPs in an *in vivo* model of acute inflammation. Both LPS mediated spontaneous loss of tone and cytokine mediated vascular dysfunction could be ameliorated by MMP inhibition. In an *in vivo* rat model of endotoxemia, net gelatinolytic activity in the aorta was increased compared to control animals. MMP-2 and MMP-9 were the gelatinases likely responsible for the increase in gelatinolytic activity. Aortic TIMP-1 was increased, TIMP-4 was decreased, and TIMP-2 remained unchanged in LPS treated rats. This is the first study to demonstrate that MMP inhibition ameliorates inflammatory vascular dysfunction, and that vascular MMPs and TIMPs are acutely regulated *in vivo* by a severe inflammatory stress.

The model of LPS-mediated spontaneous relaxation of rat aorta *in vitro* was first established by Moncada and colleagues.[17] It was found that ambient levels of LPS were high enough to cause an inflammatory response in vessels incubated for longer periods in Krebs buffer under non-sterile conditions.[17,39] The ability of polymyxin B, a known chelator of LPS,[40] to prevent relaxation suggests that the loss of tone was LPS mediated under our experimental conditions. Doxycycline was also demonstrated to prevent the loss of tone. Doxycycline is a member of the tetracycline class of antibiotics, which are well recognized MMP inhibitors.[41] Interestingly, this inhibitory effect is independent of their antibiotic actions, and it is believed that they inhibit MMPs through their ability to chelate the Zn^{2+} located in the proteinase catalytic site. A chemically distinct MMP inhibitor, GM6001, was also found to prevent the LPS mediated spontaneous loss of tone. GM6001 is thought to inhibit MMPs via direct ligation of the catalytic Zn^{2+} along with hydrogen bonding and van der Waals forces.[42,43]

Incubating vessels *ex vivo* with IL-1β is another well-established model of vascular dysfunction.[44,45] This model was used to determine whether MMP inhibitors could protect against vascular dysfunction which is not dependent on cytokine processing. This is particularly important since MMP-2 and MMP-9 have been demonstrated to cleave and activate the IL-1β precusor.[46] IL-1β is thought to be a principal player in the cardiovascular dysfunction associated with septic shock and endotoxemia since IL-1β receptor antagonists reduce cardiovascular dysfunction and mortality.[47,48] Moreover, infusion of this cytokine into normal rabbits results in marked hypotension.[49]

In the current experiments, MMP inhibition using GM6001 significantly improved the response of IL-1β treated vessels. This suggests that part of the IL-1β mediated response is dependent on MMPs. In cell culture models, IL-1β is recognized to stimulate MMP-1, -3, and -9 expression in vascular smooth muscle cells[2,50] and MT1-MMP in endothelial cells.[51] Thus, part of GM6001's protective effect could be explained by an inhibition of newly synthesized MMPs.

A well-established model of endotoxemia was used to assess whether MMP and TIMPs are regulated acutely *in vivo* during severe inflammatory stress. Previously, we have demonstrated that significant cardiovascular dysfunction exists in this model at 6 h post LPS administration. This dysfunction is characterized by hypotension, and severe cardiac depression.[32,35,52] Nitric oxide and superoxide anion, markers of circulating and cardiac peroxynitrite production, and proinflammatory cytokines are all significantly increased by 6 h following LPS administration.[32,35,53,54] Since MMPs and TIMPs are regulated by proinflammatory cytokines[21,22] and peroxynitrite[23-26] alterations in MMP activity were anticipated.

Net gelatinolytic activity was found to be increased in aortas taken from LPS treated animals. The lack of an increase in collagenolytic activity suggested that the increase in gelatinolytic activity was due to MMP-2 and –9 (the gelatinases) and not due to increases in collagenase activities (MMP-1, -8, and –13, which are also able to digest gelatin). Gelatin zymography confirmed that 92 kDa MMP-9 activity was increased in LPS aortas.

Immunoblot, however, revealed a discordance between protein levels and apparent activity of both 72 kDa MMP-2 and 92 kDa MMP-9 in zymography. This discordance might be attributable to peroxynitrite-induced activation of these enzymes. Since MMPs are synthesized as zymogens, they are usually activated through proteolytic removal of the pro-peptide domain, or through oxidant stress. In this latter mechanism, peroxynitrite disrupts the propeptide domain 'cysteine switch' and exposes the catalytic zinc site of the enzyme, thereby producing an activated 'proenzyme.'[24,25] Thus, the increase in peroxynitrite production in this model of endotoxemia may have activated both the 72 kDa MMP-2 and the 92 kDa MMP-9, without a change in molecular weight. With such activation, an increase in MMP-9 activity was detected in zymography despite unchanged protein content relative to control animals, and zymographic MMP-2 activity remained constant despite a loss in protein content relative to control animals.

The increase in aortic MMP-9 activity following LPS administration adds to the mounting evidence implicating this MMP in endotoxemia and septic shock. Previously, we have demonstrated that circulating MMP-9 activity correlated inversely with mean arterial blood pressure in endotoxemic rats.[32] As well, *in vivo* administration of MMP inhibitors (doxycycline and Ro 31-9790) significantly improved endotoxemic cardiac dysfunction, partly through a blunting of MMP-9 activation and release from the heart.[52] Dubois *et al* demonstrated that an MMP-9 knockout mouse is significantly more resistant to lethal doses of LPS.[55] In human volunteers administered with LPS, a significant increase in circulating MMP-9 activity was also noted.[30] Finally, in patients suffering from septic shock plasma MMP-9 protein levels correlated with circulating LPS levels, and were significantly higher in non-survivors than survivors.[34]

A dysregulation of aortic TIMPs was also seen with *in vivo* LPS administration. Interestingly, TIMP-4 content was significantly decreased after LPS administration. This acute drop in TIMP-4 is in contrast to another investigation in which balloon injured rat carotid arteries were found to have increased TIMP-4 protein content throughout all layers of vessel within 24 h of injury.[56] However, our observations are reminiscent of the acute loss of TIMP-4 from isolated perfused rat hearts following proinflammatory cytokine mediated cardiac dysfunction[57] and also ischemia-reperfusion injury.[3] The increase in aortic TIMP-1 was not unexpected since it is regarded as an inducible TIMP.[58] However, it should be noted that TIMP-1 inhibitory activity is adversely affected by peroxynitrite.[26] Thus, even though TIMP-1 protein content was increased its net inhibitory may not have been increased likewise.

Several limitations that exist in the present study should be noted. First, the specific target of MMP inhibition in the functional models of vascular inflammation was never established. Moreover, the potential target of MMP activity within all three models was not uncovered. As well, since the aorta is not a major site of vascular resistance the functional significance of aortic vascular response in relation to blood pressure can be questioned. Future studies will likely focus on resistance arteries and attempt to identify potential targets of MMP activity. Finally, we cannot rule out that possible post-translational modifications to the MMPs and TIMPs (under the conditions of enhanced oxidative stress) may have altered the binding affinity of the antibodies during immunoblotting.

In summary, we have demonstrated that MMPs are acutely regulated in the vasculature during severe inflammation. Moreover, MMP inhibitors could prevent vascular dysfunction in two inflammatory models. These data suggest that MMPs play a role in the vascular dysfunction associated with conditions such as endotoxemia and septic shock.

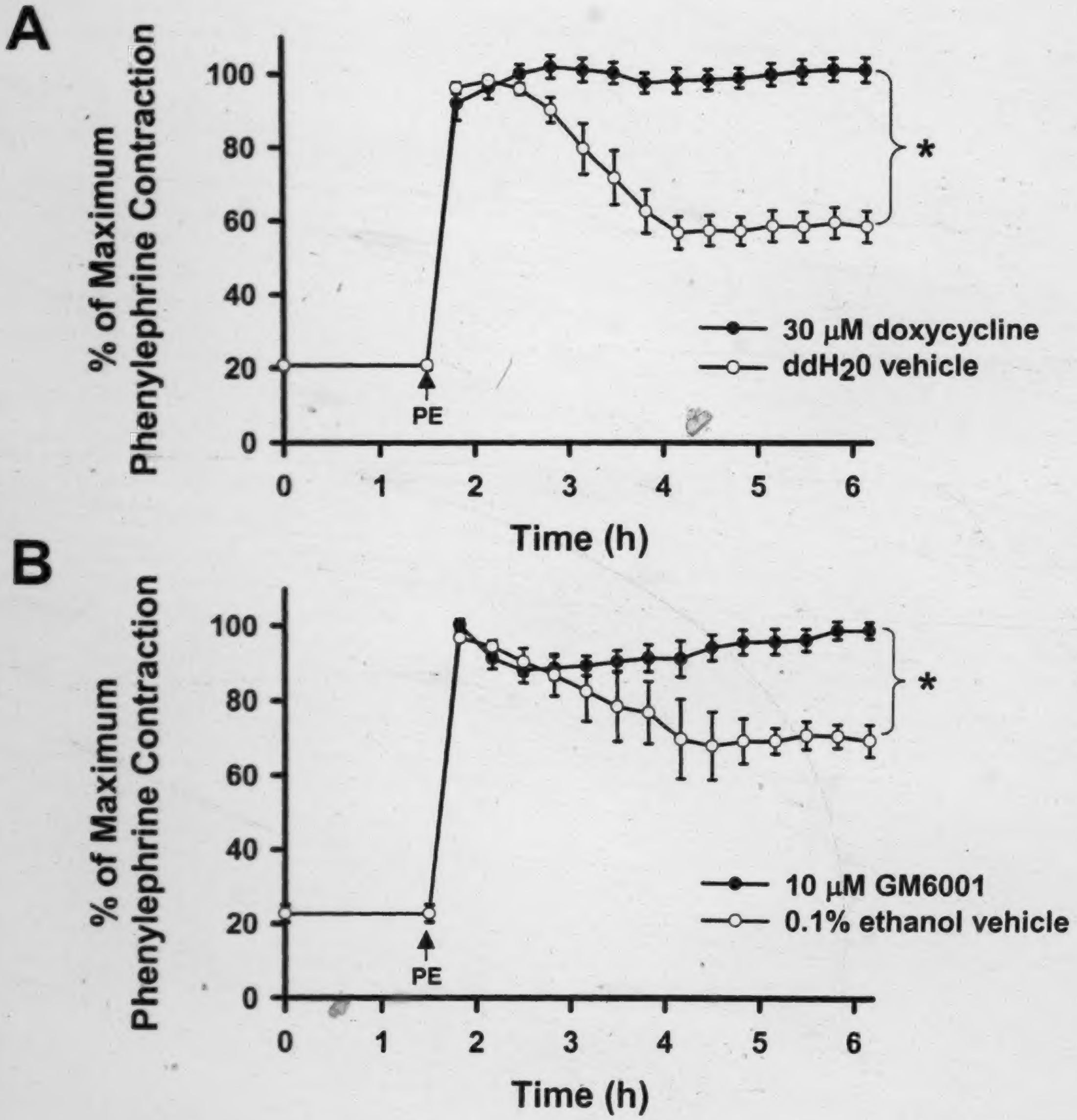

Figure 3.1 Response of aortic rings taken from normal rats to a lipopolysaccharide mediated loss of phenylephrine (PE) induced tone. PE was added after 1.5 h and then rings were treated with either (A) doxycycline (30 μM) or ddH_2O vehicle or (B) 10 μM GM6001 or 0.1% ethanol vehicle. (* $p < 0.05$, two way repeated measures ANOVA, n = 4-5 aortic rings/group) *(Experiments done with help from R. Chowdhury.)*

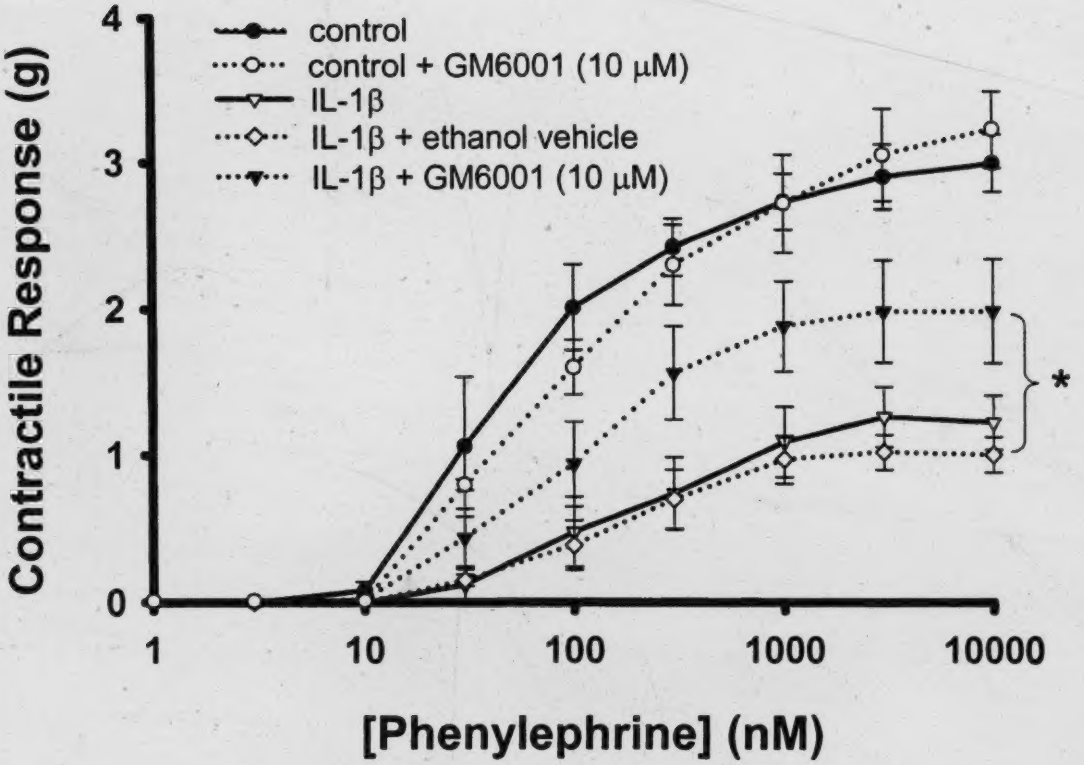

Figure 3.2 Contractile response of aortic rings taken from normal rats to increasing concentrations or phenylephrine following 6 h incubation at 37 °C in the presence or absence of IL-1β (10 ng/mL) ± GM6001 (10 μM) or its ethanol vehicle. (* $p < 0.05$, IL-1β + GM6001 vs. IL-1β + ethanol vehicle, two-way repeated measures ANOVA, n = 4 aortic rings/group) *(Experiments done with help from R. Chowdhury.)*

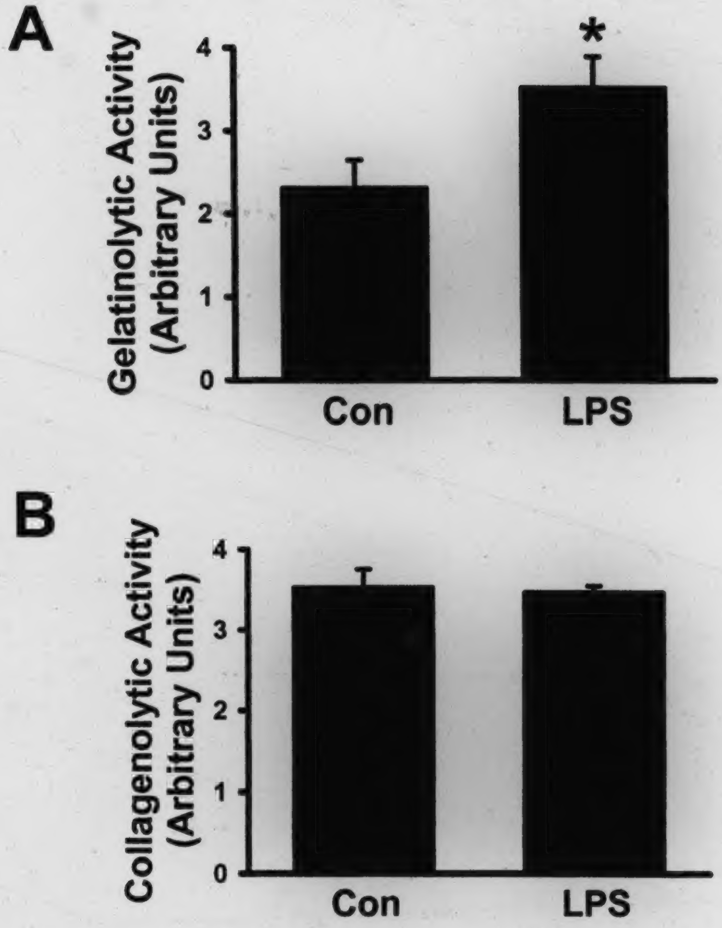

Figure 3.3 (A) Gelatinolytic and (B) collagenolytic activity of aortas taken from rats treated for 6 h with lipopolysaccharide (LPS, 4 mg/kg, i.p.) or pyrogen free water vehicle (Con) before sacrifice. (* $p < 0.05$, independent samples t-test, $n = 5$ rats/group)

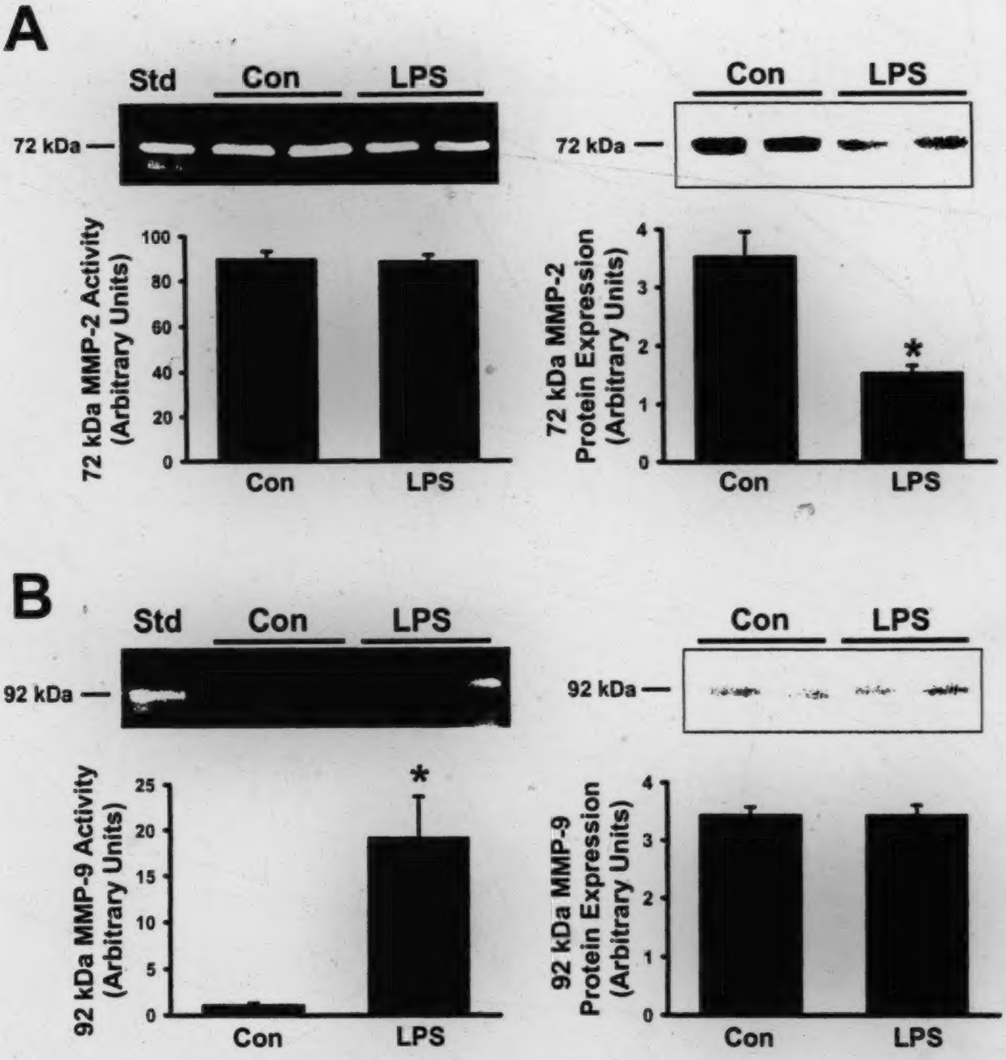

Figure 3.4 (A) Left: A representative zymogram of vascular homogenate MMP-2 activities. Aortas from two control rats (Con) and two lipopolysaccharide treated rats (LPS) primarily show 72 kDa activity. "Std" represents activities from HT-1080 cell line. Gel incubation time: 20 h. Right: a representative immunoblot showing 72 kDa MMP-2 protein content. (B) Left: A representative zymogram of vascular homogenate MMP-9 activity. 92 kDa activity appears in aortas from two LPS treated rats but not Con rats. Gel incubation time: 30 h. Right: a representative immunoblot showing 92 kDa MMP-9 protein content. (* $p < 0.05$, independent samples t-test, $n = 9$-13 rats per group)

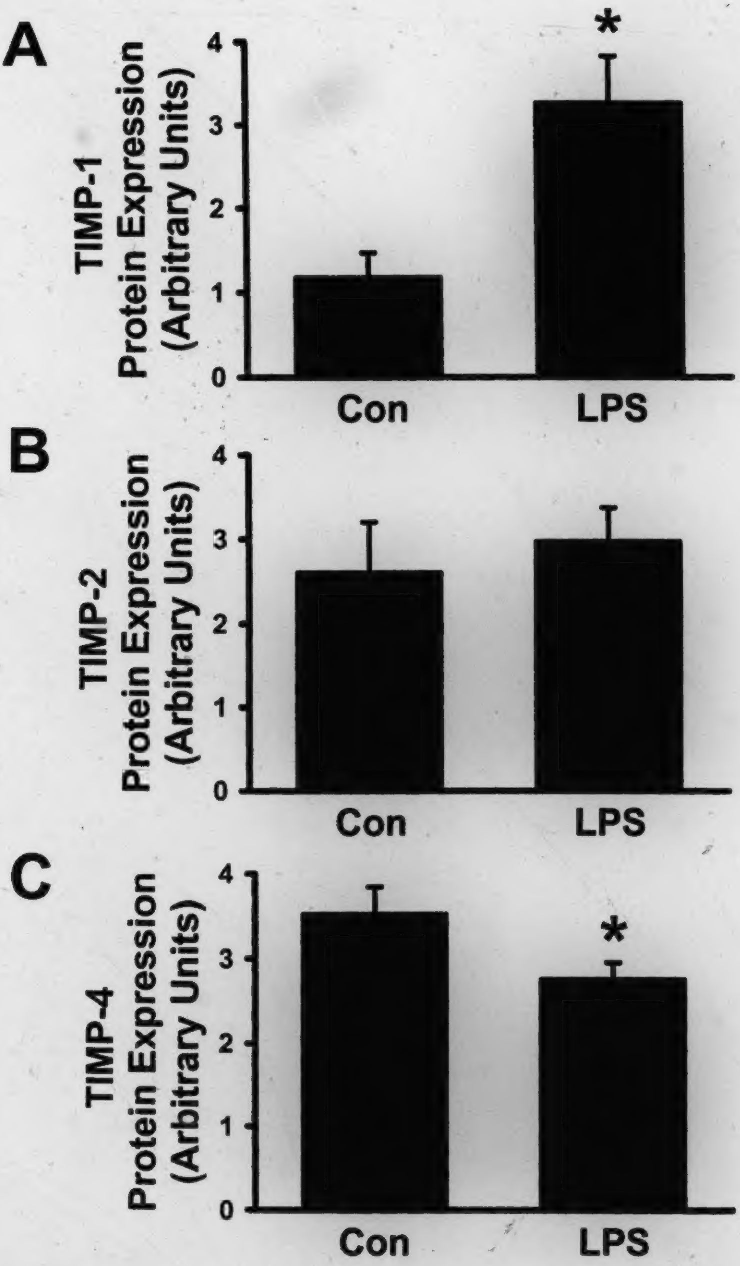

Figure 3.5 (A) TIMP-1 (B) TIMP-2 and (C) TIMP-4 protein content in aortas excised from rats treated for 6 h with lipopolysaccharide (LPS) or vehicle (Con). (* $p < 0.05$, n = 12-13 rats/group for TIMP-1 and TIMP-4, n = 6 rats/group for TIMP-2)

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CHAPTER 4

PRECONDITIONING DECREASES ISCHEMIA-REPERFUSION INDUCED RELEASE AND ACTIVATION OF MATRIX METALLOPROTEINASE-2

A version of this chapter has been published. Manoj M Lalu, Csaba Csonka, Zoltán Giricz, Tamás Csont, Richard Schulz, and Péter Ferdinandy. Preconditioning reduces ischemia-reperfusion induced matrix metalloproteinase-2 activation and release. *Biochemical and Biophysical Research Communications.* 296:937-941; 2002.

4.1 Abstract

Release and activation of matrix metalloproteinases (MMPs) significantly contributes to myocardial stunning injury immediately after ischemia and reperfusion, however, their role in preconditioning remains unknown. We therefore examined the effects of preconditioning and subsequent ischemia-reperfusion on MMP activity in isolated rat hearts.

Hearts were subjected to a preconditioning protocol (three consecutive 5 min periods of global ischemia interspersed with 5 min of reperfusion) followed by 30 min ischemia and 5 min reperfusion. In order to measure MMP release, coronary effluent was collected: a) during aerobic perfusion, b) in reperfusion following each preconditioning ischemia, and c) during the final reperfusion following test ischemia. MMP-2 activities could be detected by gelatin zymography in the ventricles and coronary effluent samples from the perfused hearts. The levels of MMP-2 activity in the effluent were markedly increased in effluent following test ischemia from control hearts without preconditioning. This was accompanied by a decrease in corresponding tissue MMP activities. Preconditioning significantly decreased the MMP-2 activity in the coronary effluent following test ischemia-reperfusion and preserved the MMP-2 protein content and activity in the myocardium. Our results demonstrate that classic preconditioning inhibits ischemia-reperfusion induced release and activation of MMP-2. These results suggest that preconditioning may exert part of its cardioprotective effects through the reduction of MMP-2 release.

4.2 Introduction

Matrix metalloproteinases (MMPs) are a large family of zinc dependent endopeptidases that are recognized for their ability to remodel the extracellular matrix. Such remodeling processes take course over periods of days to weeks. Recently, however, a number of studies have demonstrated that MMP-2 plays a major role in acute processes on the timescale of seconds to hours, such as platelet aggregation,[1] and maintenance of vascular tone,[2,3] and the regulation of inflammatory responses.[4]

MMP-2 has also been shown to be an important mediator of the acute mechanical dysfunction of the heart immediately following ischemia and reperfusion.[5] During reperfusion, MMP-2 is activated intracellularly and cleaves the contractile protein regulatory element troponin I.[6] MMP-2 is then released from the heart, likely as a means to abrogate the extent of proteolytic stress. Thus, cardiac dysfunction upon reperfusion correlates directly with the release and activation of MMP-2 from the heart.[5] This dysfunction can be ameliorated not only by the administration of non-specific MMP inhibitors but also by infusion of an anti-MMP-2 neutralizing antibody.[5]

We have also demonstrated that intracoronary infusion of peroxynitrite, the toxic reaction product of nitric oxide and superoxide, stimulates an acute release of MMP-2 from the myocardium which precedes the depression of mechanical function and mimics the effects of stunning injury.[7] Peroxynitrite induced injury could also be attenuated by the administration of MMP inhibitors.[7] These studies provide evidence that MMP-2 release and activation is a primary effector of acute cardiac mechanical dysfunction.

It has been recently shown that peroxynitrite production upon reperfusion is attenuated by ischemic preconditioning in isolated rat hearts.[8] Since peroxynitrite activates MMPs both *in vitro*,[9,10] and in the isolated heart,[7] we speculated that the decreased production of peroxynitrite in preconditioned hearts would diminish the activation and release of MMP-2. Thus, the aim of the present study was to determine the effects of ischemic preconditioning on myocardial MMP activation and release.

4.3 Materials and Methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Research Council (revised 1996) and was approved by local ethics committees.

4.3.1 Isolated heart preparation

Hearts from male Sprague Dawley rats (250-300 g, anesthetized with 100 mg/kg pentobarbital, and given 500 U/kg heparin) were isolated and briefly rinsed by immersion in ice-cold Krebs-Henseleit buffer. Spontaneously beating hearts were retrogradely perfused according to the Langendorff method at 37 °C with Krebs-Henseleit buffer as previously described.[11]

4.3.2 Experimental protocol

Hearts were either aerobically perfused in a time matched non-preconditioning ischemia-reperfusion protocol (I/R) or a no-flow ischemia induced preconditioning protocol (Preconditioning + I/R) before induction of test ischemia-reperfusion as previously described.[12] In the preconditioned group, hearts were aerobically perfused for 10 min and then subjected to three consecutive periods of global, no-flow ischemia, each of 5 min duration, interspersed with 5 min of aerobic reperfusion (Figure 5.1). Non-preconditioned hearts were perfused aerobically for 40 min. Non-preconditioned and preconditioned hearts were then subjected to 30 min global, no-flow ischemia followed by 5 min of reperfusion (test ischemia-reperfusion).

4.3.3 Collection and prepartion of coronary effluent samples

Coronary effluent samples were collected for determining MMP activities during: a) the last 2 min of aerobic perfusion, b) during the 5 min of reperfusion following each preconditioning ischemia, and c) during the 5 min of reperfusion following test ischemia. The samples were concentrated approximately 30 fold to equal volumes in Centricon 30 concentrating vessels (5000 g, 4°C, Amicon Inc, Bedford, MA) and analyzed by gelatin zymography on the same day.

4.3.4 Preparation of ventricular tissue samples

Ventricular tissue samples were taken for determining cardiac MMP activities. Immediately following the end of perfusion, the atria were cut off and ventricular tissue samples were freeze-clamped and crushed at liquid N_2 temperature by mortar and pestle. The resulting powder was diluted 1:4 w/v in 50 mM Tris-HCl (pH 7.4) buffer containing 3.1 mM sucrose, 1 mM dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin and 0.1% Triton X-100. This solution was then homogenized with an Ultra-Turrex disperser using four strokes of 4 s duration each. The homogenate was centrifuged at 10 000 g for 5 min at 4 °C and the supernatant was kept on ice for immediate assay of MMP activities.

4.3.5 Protein assay

Coronary effluent and ventricular homogenate protein contents were determined by the bicinchoninic acid method (BCA kit, Sigma) using bovine serum albumin as a standard.

4.3.6 Measurement of MMP activity by zymography

Gelatinolytic activities of MMPs were examined as previously described.[5,13] 8% polyacrylamide gels copolymerized with gelatin (2 mg/mL, type A from porcine skin, Sigma) were prepared. Non-heated samples were diluted with water in order to load a constant amount of protein per lane (coronary effluent, 2 μg; ventricular homogenate, 40 μg). An internal standard was loaded into each gel (supernatant of phorbol ester activated HT-1080 cells, American Type Culture Collection, Manassas, VA), which was used to normalize activities between gels. Following 1.5 h of electrophoresis, the gels were washed with 2.5% Triton X-100 for 1 h at room temperature (with three changes of solution) to remove sodium dodecyl sulphate. Gels were then incubated for 12 - 48 h at 37°C in incubation buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3). After incubation, the gels were stained with 0.05% Coomassie Brilliant Blue (G-250, Sigma, Oakville, ON) in a mixture of methanol:acetic acid:water (2.5:1:6.5, v/v) and destained in aqueous 4% methanol:8% acetic acid (v/v). Gelatinolytic activities were detected as transparent bands against the dark blue background. Zymograms were digitally scanned and band intensities were quantified using SigmaGel software (Jandel Corporation, San Rafael, CA) and expressed as a ratio to the internal standard.

4.3.7 Measurement of MMP-2 protein content by Western blot

Western blot analysis of issue homogenates was performed as previously described.[5] Heart extracts (40 μg protein) were applied to 7% polyacrylamide gels. Electrophoresis was carried out under reducing conditions. Samples were then electroblotted onto polyvinylidene difluoride membranes (BioRad, Hercules, CA) and probed with a rabbit anti-rat 72 kDa MMP-2 antibody (AB19015, 1:2000, Chemicon, Temecula, CA) and thereafter with horseradish peroxidase-conjugated goat anti rabbit IgG (1:5000, Sigma, Oakville, ON). The immunoreactive proteins were detected using an enhanced horse-radish peroxidase-luminol chemiluminesence reaction kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were digitally scanned and band intensities were quantified using SigmaGel software (Jandel Corporation, San Rafael, CA) and expressed as a ratio to the internal standard.

4.3.8 Statistical Analysis

Results are expressed as the mean ± SEM. All results were analyzed using SPSS software (SPSS Inc, Chicago, IL). Student's *t*-test or one way analysis of variance followed by Tukey-Kramer test were used to evaluate differences between groups. Differences were considered significant at $p < 0.05$.

4.4 Results

Gelatinolytic activities could be detected by zymography at 75, 72, and 62 kDa in the ventricles and all coronary effluents of perfused rat hearts. The 72 and 62 kDa forms were identified as MMP-2 by comparison to the HT-1080 standard. The 75 kDa band has been identified as a rat/murine specific glycosylated form of MMP-2 (personal communication, Chris Overall, University of British Columbia). MMP-9 gelatinolytic activity could be detected in neither the effluent nor the ventricles. All gelatinolytic activities were abolished by the addition o-phenanthroline (100 μM), an MMP inhibitor, to the zymography incubation buffer (data not shown).

In accordance with our previous study,[5] test ischemia-reperfusion induced a large release of MMP-2 into the coronary effluent of non-preconditioned (I/R) hearts (Figure 5.2). Preconditioning with three brief periods of no-flow ischemia significantly decreased the test ischemia-reperfusion MMP-2 activity. There were no significant changes in MMP activities during reperfusion following any of the 5 min preconditioning ischemias.

The release of MMP-2 following test ischemia-reperfusion was accompanied by a concomitant decrease in ventricular MMP-2 activity as previously described.[5] Western blot analysis of the hearts demonstrated that this loss of activity was related to the direct loss of MMP-2 protein in the non-preconditioned (I/R) hearts (Figure 5.3C). Ischemic-preconditioning of the hearts significantly attenuated this loss of MMP-2 activity and protein content (Figure 5.3).

4.5 Discussion

This is the first demonstration that ischemic preconditioning inhibits the release of MMP-2 induced by test ischemia-reperfusion.

MMP-2 is an important species of metalloproteinase which can be found in normal cardiac myocytes,[14] cardiac fibroblasts,[15] and endocardial cells.[16] MMPs are synthesized as pro-enzymes and are usually activated by proteolytic cleavage of an inhibitory pro-peptide domain. However, it has also been shown that peroxynitrite can activate this enzyme by oxidizing the sulphydryl bond between a cysteine residue of the pro-domain and the Zn^{2+} catalytic center, resulting in an active enzyme without a change in molecular weight.[10] Thus, accumulation of peroxynitrite in the reperfused heart[8,17,18] likely activated the 72 kDa ('pro-form') MMP-2 released in this investigation during reperfusion. However, the reduction of endogenous peroxynitrite formation with preconditioning[8] removed a major stimulus for MMP-2 activation during subsequent test ischemia-reperfusion, and therefore reduced the release and activation of MMP-2.

The activation and release of MMP-2 from isolated rat hearts correlates directly with the cardiac dysfunction seen with both ischemia-reperfusion injury[5] and with the infusion of peroxynitrite.[7] This dysfunction could be attenuated with the administration of several chemically distinct MMP inhibitors (doxycycline, o-phenanthroline, PD166793) and a neutralizing antibody against MMP-2.[5-7] In the model of preconditioning used in the present investigation, the reduction of MMP-2 release and activation during test ischemia-reperfusion parallels the preservation of cardiac mechanical function (left ventricular end-diastolic pressure) observed in our previous study using the same model of preconditioning.[8] Since MMP-2 cleaves troponin I during reperfusion,[6] preconditioning likely preserved cardiac mechanical function by attenuating the accumulation of peroxynitrite,[8] which subsequently reduced the activation and release of MMP-2, and diminished this proteinase's negative effects on the contractile machinery during reperfusion.

The brief periods of preconditioning ischemia used in this investigation did not significantly increase MMP-2 release from the heart. This is in accordance with our previous study which demonstrated that a global, no-flow ischemic period of at least 20 min is necessary to produce cardiac dysfunction and a significant release of MMP-2 from isolated rat hearts.[5] The lack of a significant release of MMP-2 with 5 min ischemia-reperfusion cycles, and the significant preservation of MMP-2 activity and protein content within the ventricle excludes the possibility that ischemic preconditioning merely exhausted myocardial stores of MMP-2 which led to decreased release upon test ischemia-reperfusion.

MMP-9, another MMP which is detectable by gelatin zymography, could be found in neither the ventricles nor the coronary effluent. This absence of MMP-9 activity can be explained by the lack of circulating leukocytes within our perfusion system, a major source of MMP-9. *In vivo* studies of ischemia-reperfusion injury in porcine[19] and canine[20] models demonstrated increases in MMP-9 activities within the ventricle which were ascribed to infiltrating leukocytes. Thus, the main source of MMP-9 during reperfusion was absent in the crystalloid buffer perfused system used in this investigation.

In conclusion, our present study is the first demonstration that ischemic preconditioning reduces the release and activation of MMP-2 from the heart. These results suggest that preconditioning may exert part of its cardioprotective effects through the reduction of MMP-2 release.

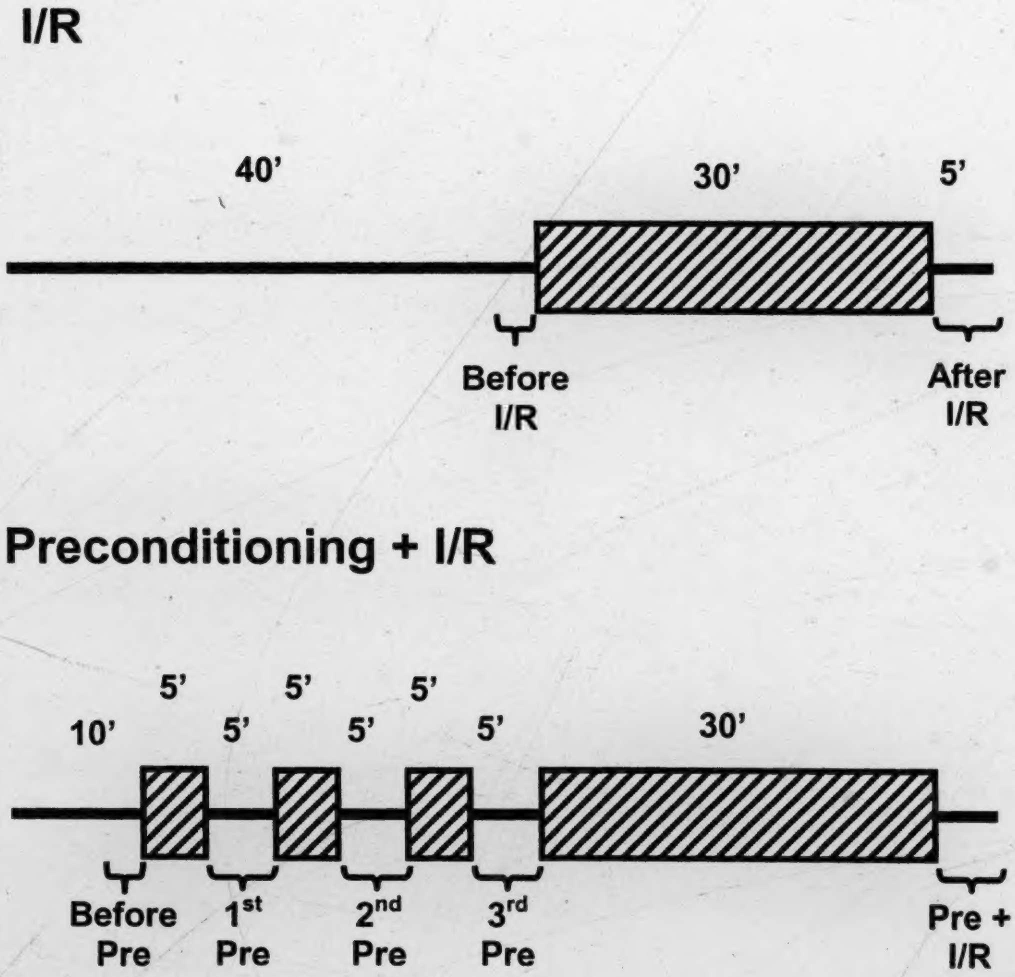
I/R
40'
30'
5'
Before I/R
After I/R
Preconditioning + I/R
10'
5'
5'
5'
5'
5'
5'
30'
Before Pre
1st Pre
2nd Pre
3rd Pre
Pre + I/R

Figure 4.1 Perfusion protocol for isolated rat hearts subjected to either no-flow global ischemia followed by reperfusion (I/R), or no-flow ischemic preconditioning followed by ischemia-reperfusion (Preconditioning + I/R). Lines indicate aerobic perfusion, and boxes indicate global ischemia. Brackets below bars indicate times of coronary effluent collection.

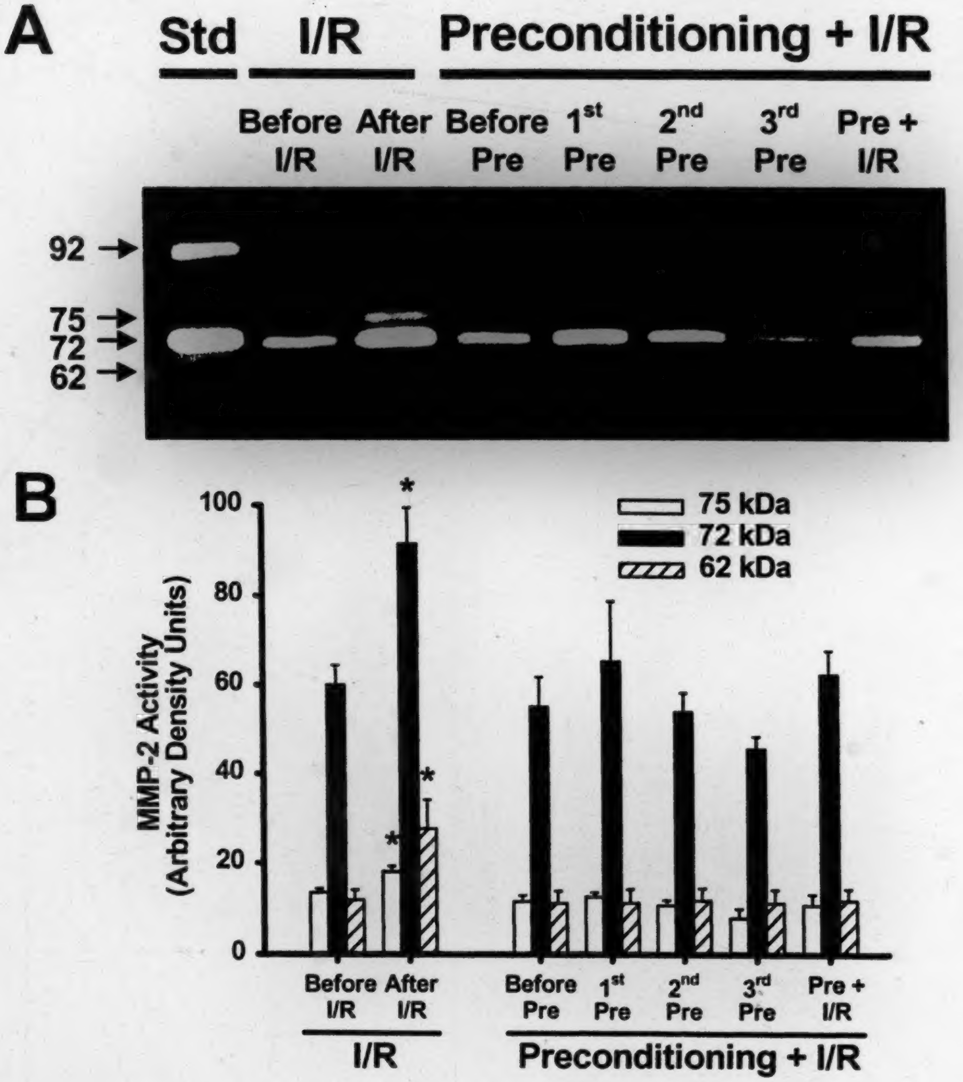

Figure 4.2 Effect of preconditioning and ischemia-reperfusion on the release of gelatinolytic activity into the coronary effluent. (A) Representative zymogram of coronary effluent samples demonstrates the presence of 75 kDa, 72 kDa, and 62 kDa MMP-2 activities. No MMP-9 activities could be detected. 'Before I/R' and 'Before Pre' indicate samples collected during the final 2 min of 40 min aerobic perfusion or the last 2 min of 10 min aerobic perfusion, respectively. 'After I/R' and 'Pre+I/R' indicates samples collected during the 5 min aerobic reperfusion. 1st, 2nd, and 3rd 'Pre' samples were collected during the 5 min aerobic perfusion following ischemic-preconditioning bursts. 'Std' indicates HT1080 cell-conditioned medium. (B) Summary data for densitometric analysis of MMP-2 gelatinolytic activities in coronary effluents. $*P<0.05$ vs. respective activities in all other groups (ANOVA). Data are the mean values of 6 samples per group, error bars represent SEM. *(Perfusion done with help from T. Csont.)*

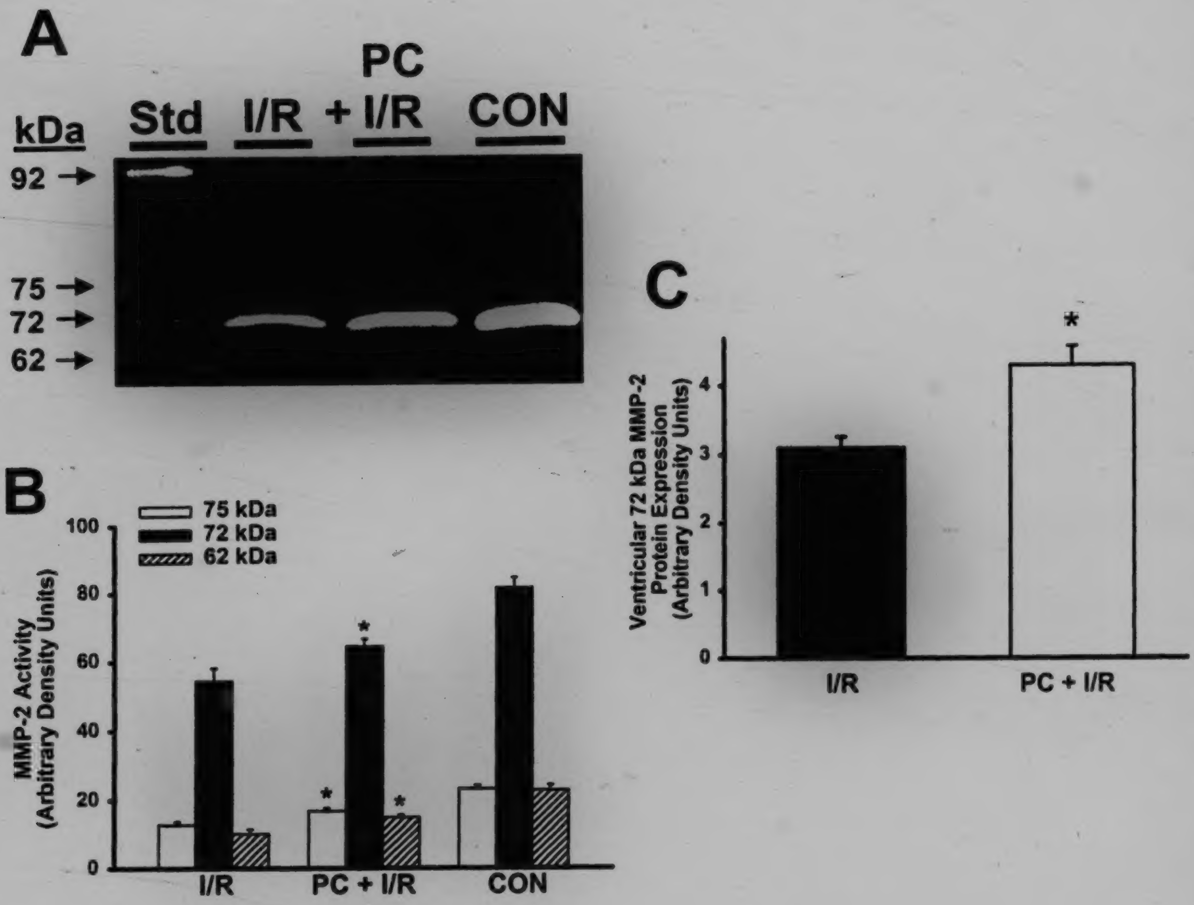

Figure 4.3 Effect of preconditioning on the gelatinolytic activities in ventricles. (A) Representative zymogram of ventricle tissue MMP-2 activities. 'I/R' indicates hearts subjected to test ischemia-reperfusion alone. 'PC + I/R' indicates heart subjected to preconditioning protocol prior to test ischemia-reperfusion. 'Con' indicates time matched aerobically perfused hearts not subjected to test ischemia-reperfusion. No MMP-9 activity could be detected. (B) Summary data for densitometric analysis of MMP-2 gelatinolytic activities in ventricles. $^*P<0.05$ vs. respective activities in ischemia-reperfusion group and in control group (ANOVA). (C) Summary data for 72 kDa MMP-2 protein level in ventricles. $^*P<0.05$ vs. ischemia-reperfusion group (Student's *t* test). Data are the mean values of 6-8 hearts, error bars represent SEM. *(Zymography done with help from Z. Giricz.)*

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CHAPTER 5

ISCHEMIA-REPERFUSION INJURY ACTIVATES MATRIX METALLOPROTEINASES IN THE HUMAN HEART

A version of this chapter has been submitted for publication. Manoj M Lalu, Evasio Pasini, Costas J Schulze, Mario Ferrari-Vivaldi, Gianna Ferrari-Vivaldi, Tiziana Bachetti, and Richard Schulz. Ischemia-reperfusion injury activates matrix metalloproteinases in the human heart. *Circulation.*

5.1 Abstract

Matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) are key regulators of matrix degradation, remodeling, and repair in the heart. It has been demonstrated that MMPs play a pivotal role in myocardial dysfunction immediately following ischemia-reperfusion injury *ex vivo* in rats. However, their role in the acute impairment of contractile function in humans remains unknown.

Fifteen patients with stable angina undergoing coronary artery bypass graft surgery were enrolled. Cardiac biopsy samples were obtained at the start of bypass before cardioplegia and within 10 minutes after removal of the aortic cross-clamp. Gelatin zymography revealed a marked increase in 72 kDa MMP-2 and 92 kDa MMP-9 activities. Western blot revealed a decrease in TIMP-1 upon reperfusion. The increase in myocardial MMP activity was positively correlated with the duration of cross-clamp and inversely with cardiac mechanical function 3 hr following reperfusion. The level of TIMP-1 correlated inversely with cross clamp time and positively with cardiac mechanical function. Zymographic analysis of plasma samples collected from the radial artery and coronary sinus revealed a significant increase in both 92 kDa MMP-9 and 64 kDa MMP-2 activities in the plasma 1 min following removal of cross-clamp, which remained elevated 10 min into reperfusion. However, no significant transcardiac differences in MMP activity existed at any time point of reperfusion.

These results demonstrate that reperfusion following cardioplegia stimulates an acute activation of MMPs in the myocardium and plasma of patients undergoing coronary artery bypass grafting. This is the first correlation of MMP myocardial activities with cardiac function in humans. The early increase in MMP activity could produce a

proteolytic environment which may play a key role in myocardial stunning injury in humans.

5.2 Introduction

Coronary artery bypass graft surgery (CABG) with cardiopulmonary bypass (CPB) is associated with stunning injury following reperfusion of the ischemic myocardium.[1] Numerous cellular mechanisms have been proposed to explain this reversible impairment of cardiac mechanical function including: 1) alterations in cardiac metabolism [2], 2) the production of reactive oxygen species,[3] 3) alterations in calcium handling,[4] and 4) the activation of myocardial proteinases.[5]

Matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases which are synthesized as zymogens. Of particular interest in the heart are the gelatinases MMP-2 (72 kDa and 62 kDa) and MMP-9 (92 kDa and 84 kDa) as they are found in cardiac myocytes,[6] cardiac fibroblasts,[7] and endocardial cells.[8] The activity of these MMPs can be regulated by the tissue inhibitors of metalloproteinases (TIMPs), four of which have been identified thus far in humans. TIMP-4 is the most abundant in the human heart.[9] They are all able to inhibit a broad variety of MMPs.[10]

MMP-2 and -9 are well recognized for their proteolytic action on extracellular matrix proteins and their involvement in long term remodeling processes which occur in several physiological as well as pathological events such as heart failure.[11-13] and atherosclerosis.[14] However, recent data has implicated MMP-2 in a variety of acute physiologically relevant processes on novel substrates unrelated to the extracellular matrix. These include platelet aggregation,[15] regulation of vascular tone,[16,17] and modulation of the inflammatory response.[18]

Stunning injury following reperfusion of the ischemic myocardium is another acute process in which MMPs have been implicated. We have shown that MMP-2 is directly involved in acute stunning injury seen during reperfusion following ischemia in isolated rat hearts.[19] During reperfusion, MMP-2 is activated intracellularly and cleaves the contractile protein regulatory element troponin I.[20] MMP-2 is also released from the rat heart during acute reperfusion, likely as a means to abrogate the extent of injury, and inhibition of MMP activity significantly improves cardiac function during reperfusion.[19,20] Most of these actions occur on a rapid time scale (seconds to minutes) where MMPs could be thought to have actions as signaling proteinases with detrimental effects on cardiac mechanical function.

To date, no data is available on the role of MMPs and TIMPs in heart function in patients following ischemia-reperfusion injury. This study was designed to investigate in the clinical setting of CABG with CPB whether MMPs are activated and/or released by the myocardium and whether changes in MMP and TIMPs are related to post-ischemic myocardial function.

5.3 Materials And Methods

5.3.1 Patient Population

Fifteen patients with stable angina undergoing elective CABG surgery were recruited at the San Rocco Hospital between 09/2000 - 01/2001 and 07/2002-09/2002. This study was approved by the institutional ethics committees at the San Rocco Hospital and the University of Alberta. All patients gave informed consent. Exclusion criteria for the study were: a) reduced ejection fraction, b) previous myocardial infarct, c) enlargement or hypertrophy of cardiac chambers, d) concomitant valvular diseases, e) arrhythmia, f) metabolic disorders, and g) concomitant liver, pulmonary, or kidney diseases. All patients were receiving calcium channel blockers and β-blockers and all oral medication was discontinued 2 days prior to surgery. All patients underwent multiple isolated aortocoronary bypass grafts with internal mammary arteries and saphenous veins (n =2-4).

5.3.2 Surgical Procedure

In all patients anaesthesia and muscle relaxation was induced with propofol (2.0-2.5 mg/kg) and atracurium (0.08 mg/kg), respectively. Propofol was continuously infused (4-6 mg/kg/h) in order to maintain anesthesia. Ventilation was controlled with 50% O_2 in air. Before sterniotomy, an 18 gauge cannula was placed in the radial artery for arterial sampling. A Swan-Ganz catheter was introduced through the left jugular vein for haemodynamic measurements. After median sterniotomy, the aorta, inferior vena cava, and coronary sinus were cannulated and heparin (3 mg/kg) was administered. CPB was conducted with non-pulsatile flow at a rate of 2.4 L/min/m^2. The aorta was cross-clamped and intermittent antegrade and retrograde warm blood cardioplegia was used as previously described.[21] Cardioplegia was initiated through the infusion of whole blood (flow rate 300 mL/min) plus 20 mEq/L of K^+ (flow rate 2 mL/h). The cardioplegic solution was injected at 37°C for 2 min into the aortic root and then into the coronary sinus. This dose of cardioplegic solution was injected every 15-20 min. CPB was maintained with moderate hemodilution (hematocrit 24%-33%).

After completion of distal anastomoses, the aortic cross-clamp was removed and the construction of the proximal anastomoses was begun. At the end of the grafting procedure, protamine (3 mg/kg) was injected to reverse the effect of heparin. Inotropic drugs were not used.

5.3.3 Haemodynamic Measurements

Left ventricular stroke work index (LVSWI) was used as a measure of global left ventricular function.[22] Haemodynamic measurements were performed in the operating theater before starting CPB, and 1, 3, 6, 12, and 24 h after cross-clamp release.

5.3.4 Tissue and Blood Sampling

Tissue biopsy samples were sampled by one surgeon only. Immediately after the start of CPB, but before cardioplegia, a biopsy sample (approximately 15 mg wet weight) was obtained from the right atrium. Ten minutes after the release of the aortic cross-clamp, another biopsy sample was taken from the right atrium as close as possible to the previous one. No clinical complications resulted from the procurement of these biopsy samples. Biopsies were collected in cryovials, immediately frozen in liquid N_2 in the operating theater, and then stored at –80 °C.

Prior to surgery 4 mL of fresh venous blood was collected from the antecubital vein. During surgery, 4 mL of radial artery (arterial) and coronary sinus (venous) blood were collected simultaneously before CPB and 1, 5, and 10 min following cross-clamp release. Blood samples were immediately centrifuged at 3000 g for 10 min at 4 °C. The plasma fraction was then removed, frozen in liquid N_2, and stored at –80 °C.

5.3.5 Preparation of Biopsy Samples

Myocardial samples were crushed at liquid N_2 temperature, then homogenized by sonication in 50 mmol/L Tris-HCl (pH 7.4) containing 3.1 mmol/L sucrose, 1 mmol/L dithiothreiotol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin, and 0.1% Triton X-100. The homogenate was centrifuged at 10 000 g at 4 °C for 10 min, and the supernatant was collected and stored at –80 °C until use.

5.3.6 Measurement of MMP-2 and -9 Activities by Zymography

20 μg of either myocardial homogenate or plasma was loaded onto nondenaturing 8% acrylamide gels copolymerized with 2 mg/mL of gelatin, electrophoresed, renatured, and developed as previously described.[19,23] To quantify the activity of detected gelatinases, zymograms were digitally scanned, and band intensities were analyzed using SigmaGel software (Jandel Scientific). Intensities were normalized to an internal standard (supernatant from HT-1080 human fibrosarcoma cell culture) loaded in each gel.

5.3.7 Measurement of Collagenase Activity

In order to measure the activities of collagenases (MMP-1, -8, and -13), 50 μg of myocardial homogenates were analyzed using an MMP collagenase activity assay kit (ECM kit 710, Chemicon) according to the manufacturer's instructions. The samples, however, were not chemically treated to activate latent collagenase activity.

5.3.8 Immunoblot analysis

20 μg of either myocardial homogenate or plasma was loaded onto 8% acrylamide gels, electrophoresed under reducing conditions, and then electroblotted onto

polyvinyllidene difluoride membranes (BioRad). Samples were probed with either a mouse anti-human MMP-2 that has a higher affinity for the 72 kDa form (1:200 dilution, MAB13405, Chemicon), a mouse anti-human MMP-2 antibody that has a higher affinity for the 64 kDa form (1:1000 dilution, MAB3308, Chemicon), a rabbit anti-mouse MMP-9 antibody (1:1000 dilution, AB19047, Chemicon), a mouse anti-human TIMP-1 antibody (1:133 dilution, MS608, NeoMarkers), a rabbit anti-human TIMP-2 antibody (1:133 dilution, RB1489, NeoMarkers), or a rabbit anti-human TIMP-4 antibody (1:4000 dilution, AB816, Chemicon). Samples were subsequently probed with appropriate horse-radish peroxidase conjugated antibodies (either anti-mouse or anti-rabbit, Transduction Laboratories), and visualized using the enhanced horse-radish peroxidase-luminol chemiluminesence reaction kit (Amersham Pharmacia Biotech).

5.3.9 Protein Assay

Protein concentrations were assessed by the bicinchoninic acid assay (Sigma) with bovine serum albumin as a standard.

5.3.10 Statistical Analysis

Data are expressed as mean ± SEM. One-way repeated measures ANOVA or paired samples t-test were used as appropriate. Correlation between values was performed using linear regression. A value of $p < 0.05$ was considered statistically significant.

5.4 Results

5.4.1 CABG Surgery with CPB Produces Reversible Cardiac Mechanical Dysfunction

Table 6.1 provides descriptions of the patients and the operative procedures. All patients in this study tolerated the surgical procedures and survived without complications. CABG surgery with CPB produced a significant acute depression in post-operative cardiac mechanical function as measured by LVSWI (Figure 6.1). This depression was maximal at 3 h post reperfusion. Cardiac function slowly returned to baseline levels and by 24 h was no longer significant versus baseline.

5.4.2 MMP-2 and –9 Activities are Increased in the Reperfused Myocardium

Gelatinolytic activities were detected at 92 kDa and 72 kDa in the myocardium prior to aortic cross-clamping (Figure 6.2A). When gels were incubated for a longer period of time, weak gelatinolytic activities could also be detected at 135 and 64 kDa prior to ischemia. 92 kDa activity was identified as MMP-9, and 72 and 64 kDa activities were identified as MMP-2 by comparison to the HT-1080 standard. The 135 kDa band likely represents a lipocalin associated MMP-9.[24] Quantitative analysis of the zymograms demonstrated that the rank order of gelatinolytic activity prior to ischemia was 92 kDa > 72 kDa > 135 kDa > 64 kDa. There was no significant linear correlation between 92 and 72 kDa activities in the preischemic biopsy sample and preischemic LVSWI (data not shown).

Within 10 min of release of the aortic cross-clamp and reperfusion, myocardial 135 and 92 kDa MMP-9 and 72 kDa MMP-2 activities were markedly elevated without any change in 64 kDa MMP-2 activity (Figure 6.2B, 2C, 2D, 2E). Post-ischemia there was a significant inverse correlation between 92 kDa MMP-9 and 72 kDa MMP activities in the reperfused biopsy sample and LVSWI at the time (3 h) when cardiac function was most severely depressed (Figure 6.3A, $p<0.05$). A significant positive correlation existed between 92 kDa and 72 kDa activities and aortic cross-clamp duration (Figure 6.3B, $p<0.05$). No correlation existed between any of the MMP activities and the duration of CPB (data not shown). Thus, elevated levels of MMP-2 and –9 activities in the biopsies taken within 10 min of reperfusion predicted a lower left ventricular function 3 h into reperfusion, a time when ventricular function was at its worst. Moreover, the longer the duration of cross-clamp (ischemic) time, the higher the MMP activities upon reperfusion.

Net myocardial collagenase activity (a composite of MMP-1, -8 and –13 activities) was measured in atrial samples from six patients. Prior to ischemia collagenase activity could be detected (1.8 ± 0.8 arbitrary activity units) and after reperfusion this was significantly increased (3.7 ± 1.2 arbitrary activity units, $p<0.05$). Post reperfusion collagenase activity, however, did not correlate with any clinical parameters.

5.4.3 Increases in Myocardial MMP Activities are Not Related to Increased MMP Protein Levels

Western blot analysis of MMP-2 and MMP-9 in the myocardial biopsies was performed in order to determine whether increases in MMP activity could be attributable to increased protein levels in the myocardium. Two antibodies were used to detect MMP-2, one which was more selective for the 72 kDa form, and another which was more selective to the 64 kDa form. Both bands could be detected prior to ischemia (Figure

6.4B and C). Upon reperfusion, the 72 kDa band remained unchanged, whereas the 64 kDa band decreased by ~33% (Figure 4B and C). Immunoblot was also performed to detect 92 kDa MMP-9. The protein content of this form of MMP-9, like the 72 kDa MMP-2, remained unchanged after reperfusion (Figure 4A).

Myocardial levels of TIMPs-1, -2 and -4 were measured by western blot. The level of TIMP-1 was significantly decreased following reperfusion (4.4 ± 1.2 arbitrary units preischemia versus 1.2 ± 0.3 arbitrary units post-ischemia, $p<0.05$, $n=6$). Moreover, post-ischemic levels of TIMP-1 correlated positively with LVSWI at 3 h ($r=0.931$, $p=0.007$) and inversely with ischemic duration ($r=-0.934$, $p=0.006$). Levels of TIMP-2 ($n=6$) and TIMP-4 ($n=15$) remained unchanged following ischemia and reperfusion (data not shown).

5.4.4 MMP-2 and –9 Activities in the Plasma are Increased Following CPB

Our previous studies in isolated rat hearts subjected to global, no-flow ischemia and reperfusion showed a marked release of MMP-2 into the perfusate which peaked 2-5 min after reperfusion.[19,20] In order to determine whether the human myocardium was releasing MMPs, plasma samples from the coronary sinus and radial artery were collected prior to aortic cross-clamp and 1, 5, and 10 min following reperfusion. Prior to ischemia, but after the onset of CPB, weak 135 kDa and 92 kDa MMP-9 activities were detected in the plasma, along with 72 kDa MMP-2 (Figure 6.5A). The predominant activity was 72 kDa MMP-2. When the gels were incubated for a longer period of time, 84 kDa MMP-9 and 64 kDa MMP-2 activities could be detected (Figure 6.6A).

Within one minute of reperfusion, 135 kDa MMP-9 and 92 kDa MMP-9 activities were significantly increased (Figure 6.5B and D, $p<0.05$). As well, 64 kDa MMP-2 increased following reperfusion (Figure 6.6C, $p<0.05$). These MMP activities were stably elevated over the first 10 min of reperfusion ($p<0.05$ for all increases in activity at all time points of reperfusion). Increases in activity were accompanied by significant increases in the protein level of 64 kDa MMP-2 and 92 kDa MMP-9 in the plasma as measured by immunoblot following reperfusion (data not shown).

There were no detectable transcardiac differences in any of the plasma MMP activities (i.e. differences between the arterial and venous samples) following reperfusion. The increases in activity did not correlate with changes in cardiac function, cross-clamp time, or cardiopulmonary bypass time (data not shown). As the surgical procedure itself could possibly alter plasma MMP activities we compared the samples taken during surgery (preischemia) with those taken from the antecubital vein before the patient went into surgery. Of the MMP activities, only 92 kDa MMP-9 activity was found to be significantly increased (13 ± 9 arbitrary units presurgery versus 49 ± 23 arbitrary units in the coronary sinus preischemia and 36 ± 17 arbitrary units in the radial artery preischemia, $p<0.05$, $n=6$).

5.5 Discussion

We studied the expression and activity of MMPs and TIMPs in the myocardium and MMPs in the plasma of patients undergoing CABG with CPB. During reperfusion following warm blood cardioplegia there was a rapid (within 10 min) increase in MMP-2, MMP-9, and collagenase activities in the myocardium which was accompanied by a loss in TIMP-1. The enhanced MMP-2 and -9 activities and the loss of TIMP-1 correlated to the post-bypass impairment of left ventricular global function seen at 3 h reperfusion, as well as to the duration of ischemia. We also demonstrated that MMP activity in the plasma was increased following CABG with CPB. This is the first study, of either acute or long-term cardiac pathologies, to clearly correlate MMP activity in the human heart to cardiac function.

5.5.1 MMP-2 and MMP-9 in cardiovascular pathology

The gelatinases were chosen as the focus of this investigation as MMP-2 is ubiquitously expressed throughout the body[25] and MMP-9 is a cytokine inducible MMP.[26] MMP-2 activity is increased in the myocardium of spontaneously hypertensive heart failure rats and inhibition of MMP-2 activity ameliorated ventricular remodeling and dysfunction.[27] Targeted deletion of MMP-9 attenuated left ventricular remodeling after experimental myocardial infarction in mice.[28] As well, both MMP-2 and MMP-9 activities are increased in the myocardium of patients suffering from dilated cardiomyopathy.[27] Clearly, these studies provide evidence that MMP-2 and MMP-9 are involved in the development of long-term heart failure.

Other investigations have established MMPs as mediators of the acute (minutes to hours time scale), reversible contractile depression following ischemia-reperfusion injury. We demonstrated that MMP-2, the most robust gelatinase activity in the rat myocardium, is acutely activated and released during the first minutes of reperfusion from isolated rat hearts subjected to global, no-flow ischemia.[19,20] The activation of MMP-2 in the heart correlated directly with ischemic time and inversely with the recovery of mechanical function following reperfusion. Moreover, MMP inhibitors or a neutralizing MMP-2 antibody attenuated mechanical dysfunction following reperfusion.[19] Myocardial MMP-1[29] and MMP-9[29,30] activities have also been shown in animal models to be upregulated some hours into reperfusion following ischemia *in vivo*. As well, targeted deletion of MMP-9 in mice was found to decrease infarct size following no-flow ischemia-reperfusion injury.[31] In the present investigation, we have demonstrated that activation of MMP-2 and –9 in the human myocardium correlated with the severity of the ischemic insult and the resulting mechanical dysfunction. These results extend previous experimental findings in animal models to the clinical setting, and raise the possibility that MMP inhibition may be a novel therapeutic strategy to prevent stunning injury in humans.

Patients without preexisting left ventricular dysfunction were selected for this study, thus, the resulting post reperfusion cardiac dysfunction was mild and reversible. Studies have demonstrated that patients with preexisting ventricular dysfunction who are subjected to cardiac revascularization represent a high mortality risk group.[32] It is possible that MMP activation would be enhanced in these patients and ultimately contribute to adverse clinical outcomes (postoperative myocardial infarction, ventricular failure, cardiac death).

5.5.2 Acute regulation of MMP activity in the human heart

MMP activity in cells can be regulated at the transcriptional level, postranslationally, and through inhibition by endogenous inhibitors (TIMPs). Proinflammatory cytokines, elevated following bypass surgery,[33] have been shown to increase MMP transcription *in vitro*.[7,34,35] However, this mechanism cannot account for the rapid increase in myocardial MMP activity seen in the present study since MMP protein levels were not elevated. The absence of increased protein levels also excludes neutrophil infiltration as a possible source for the increased 92 kDa and 72 kDa MMP activities, a notion supported by a previous study which demonstrated negligible infiltration post bypass.[36] However, the 135 kDa MMP-9 may have been derived from a neutrophil source, as this protein has only been characterized thus far in human neutrophils.[24]

Since MMPs are synthesized as zymogens, they must be activated postranslationally through proteolytic removal of the pro-peptide domain, or through modification by oxidant stress.[37,38] In this latter mechanism, the powerful endogenous oxidant peroxynitrite disrupts the MMP propeptide domain 'cysteine switch' and exposes the catalytic zinc site of the enzyme, thereby producing an activated 'proenzyme'. Thus, an increase in myocardial peroxynitrite production which occurs immediately upon reperfusion of the ischemic rat heart[3] or in human hearts during bypass surgery[39] may increase MMP activity without a loss of the propeptide domain or an increase in protein levels. This provides a possible explanation for the discordance between MMP activity and protein levels noted in this study. We have demonstrated that peroxynitrite infusion into the isolated rat heart activates myocardial MMPs without a change in molecular weight immediately prior to the onset of the mechanical dysfunction, and that inhibition of MMP activity prevented the peroxynitrite-induced acute loss in myocardial mechanical function.[40] Thus, MMPs may be terminal effectors of the increased oxidative stress which has been noted post-bypass.[41]

TIMPs provide another level of regulation of MMP activity by complexing with these enzymes and inhibiting their activity. We found a significant decrease in myocardial TIMP-1 levels which positively correlated with ischemic time, and inversely correlated with cardiac function. This finding is supported by previous studies which demonstrated decreased myocardial TIMP-1 mRNA expression following acute ischemia-reperfusion injury in isolated rabbit hearts[42] and decreased serum levels of TIMP-1 following acute myocardial infarction in humans.[43] In contrast to the changes we found in myocardial TIMP-1, there were no significant changes in TIMP-2 and TIMP-4 levels following bypass. Thus, at the same time that MMP activities are increased in the heart, the level of their primary inhibitors are either decreased or unchanged, resulting in a possible imbalance between MMPs and TIMPs and increased proteolysis. Moreover, the inhibitory activity of TIMPs following ischemia-reperfusion may be acutely diminished as peroxynitrite has also been shown to inactivate them.[44]

5.5.3 Potential targets for MMP activity

Most studies have ascribed the deleterious effects of MMPs to their ability to degrade the components of the extracellular matrix (ECM).[12,13,36] However, in the acute time frame of ischemia reperfusion injury, it is unlikely that sufficient collagen degradation occurs to contribute to stunning injury. Thus, MMPs may degrade non-ECM substrates to produce acute mechanical dysfunction. MMP-2 has been shown to cleave a number of non-ECM substrates, such as endothelin,[16] platelet adhesion receptors,[15] and monocyte chemoattractant protein.[18] Interestingly, MMP-2, MMP-9, and MT1-MMP were found to be closely associated with the sarcomeres in human cardiomyocytes.[11,45] This indicates that the contractile machinery represents a potential molecular target for the detrimental actions of MMPs in the myocardium. We recently demonstrated that MMP-2 colocalizes with the contractile protein regulatory element troponin I within sarcomeres and causes stunning injury in ischemic-reperfused rat hearts by degrading troponin I.[20] Thus, MMP activation seen in the present study may contribute to troponin I proteolysis[46] or the deranged actin cross striation pattern[47] following stunning injury in humans. Alternatively, MMP activation may have potentially beneficial effects in the reperfused myocardium. MMPs may release growth factors and cell surface receptors which could accelerate healing.[48,49]

5.5.4 MMP activity in the plasma following CABG with CPB

A previous report[50] demonstrated that a large portfolio of MMPs are elevated in the plasma by CPB, however, only protein levels and not activity were examined. In our study we have found that the surgical procedure itself increased plasma 92 kDa MMP-9 activity even prior to ischemia. Reperfusion further increased this activity, along with 135 kDa MMP-9 and 64 kDa MMP-2 activities. The increases in plasma 92 kDa MMP-9 and 64 kDa MMP-2 activities were accompanied by increases in the protein content for both. This suggests that neutrophils may have been a potential source of this increased plasma activity, as white blood cells contain large stores of MMPs.[30] Alternatively, platelet activation may have contributed to the increased plasma MMP activity.[51] As these cells came in contact with the foreign surfaces of the extracorporeal circulation they likely became activated and degranulated, liberating MMPs from intracellular stores into the plasma. Although the increase in plasma MMP activities did not correlate with any clinical measures, it likely contributes to an elevated systemic proteolytic state that may alter cardiovascular homeostasis in the postoperative period. MMPs may cause post-reperfusion coronary artery vasoconstriction through the activation of endothelin,[16] stimulate platelet aggregation[15] and contribute to coagulation disorders and the diffuse inflammatory state seen following bypass surgery.

Surprisingly, no transcardiac release or uptake of MMPs was noted in our patients. We chose early time points as we previously demonstrated that MMP-2 activation and release in rat hearts was significantly increased in the early minutes of reperfusion.[19,20] The absence of a detectable release of MMPs may have occurred for two reasons. First, the additional volumes of cardioplegic solution that were injected every 15-20 min may have washed out the proteins. However, this hypothesis is unlikely because very low volumes of cardioplegic solution were used. Second, MMP release from the myocardium may have been delayed. Indeed, 72 kDa MMP-2 protein has shown to be elevated in plasma of CABG patients only 6 to 24 h post CPB.[50]

5.5.5 Limitations

A number of limitations of these results should be recognized. First, MMP activities from atrial biopsy samples were correlated with left ventricular function. Atrial tissue was sampled due to ethical limitations, to sample an appropriate amount of tissue without damaging the left ventricle from multiple needle biopsies, and to avoid taking biopsies from a potentially ischemic portion of left ventricle. Second, a limited number of patients were enrolled in this study. Finally, the effects of ischemia-reperfusion may be enhanced by the effects of cardiopulmonary bypass. However, in this study myocardial MMP activity correlated with ischemic time and not cardiopulmonary bypass time.

5.5.6 Clinical implications

Our findings in patients undergoing CABG with CPB indicate that MMPs are activated when the human myocardium is subjected to ischemia-reperfusion. The increase in the activity of these enzymes is likely to produce a proteolytic environment within the patient which may contribute to post-surgical complications, including myocardial stunning injury. Based on the results of this investigation, the inhibition of MMPs or enhancement of TIMPs may prove to be a novel therapeutic strategy to prevent acute postoperative cardiac dysfunction.

Table 5.1. Patient demographics

Surgical Variables	
Bypass time, min	61.3 ± 4.1
Cross-clamp time, min	31.3± 1.9
Number of grafts (range)	2.7 ± 0.2 (2-4)
Pacing used, n	0
Preoperative systolic pressure, mmHg	120.0 ± 1.8
Postoperative systolic pressure, mmHg	113.0 ± 1.8
Cardiac ICU duration, h	41.6 ± 0.9
Preexisting conditions	
Age, y	60.9 ± 1.4
Male, n	12
Female, n	3
Hypertension, n	9
Hyperlipidemia, n	6
Family history of cardiac illness, n	4
Smokers	14

Mean ± SEM values are given.

(Data collected by E. Pasini.)

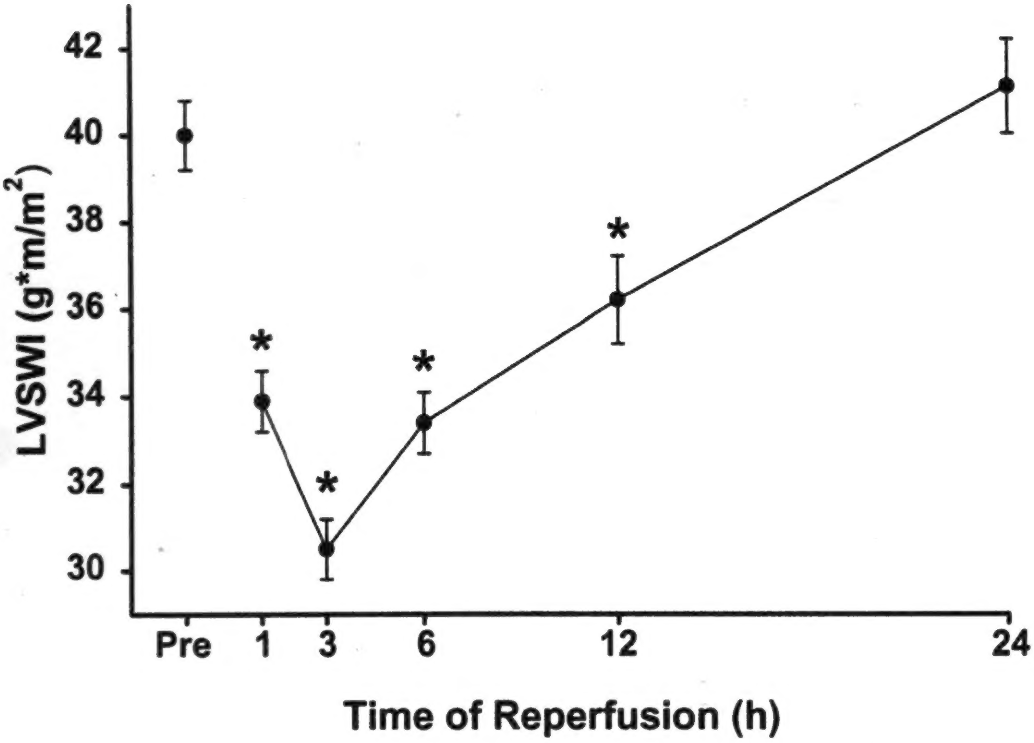

Figure 5.1 Cardiac mechanical function in patients prior to cardioplegia (Pre), and post reperfusion. Left ventricular stroke work index (LVSWI) was monitored for 24 h post reperfusion. *$p<0.05$ vs. Pre, n=15 *(Data collected by E. Pasini.)*

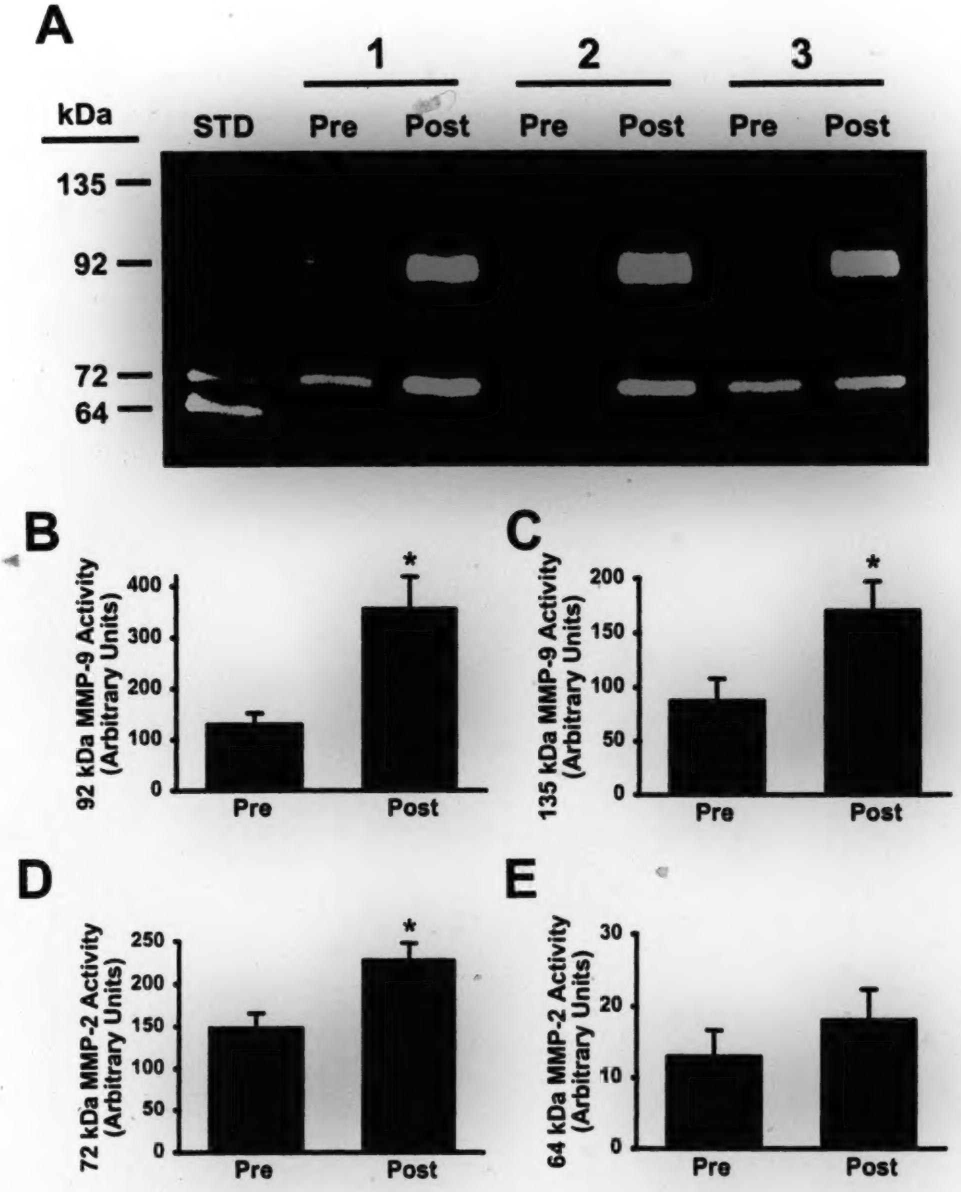

Figure 5.2 MMP activities in myocardial biopsies prior to (Pre) and following reperfusion injury (Post). Pre samples were obtained after the initiation of CPB but prior to cardioplegic arrest, and post samples were taken within 10 min of reperfusion. (A) Representative zymogram of myocardial MMP activities in three patients. HT-1080 cell culture medium was loaded into the left lane as a standard (STD). The next three sets of lanes represent myocardial MMP activities in biopsies from three patients. 135 kDa MMP-9, 92 kDa MMP-9, 72 kDa MMP-2, and 64 kDa MMP-2 activities could be detected. (B – E) Gelatinolytic activities were quantified by densitometric analysis (* $p<0.05$ vs. Pre value, n=15).

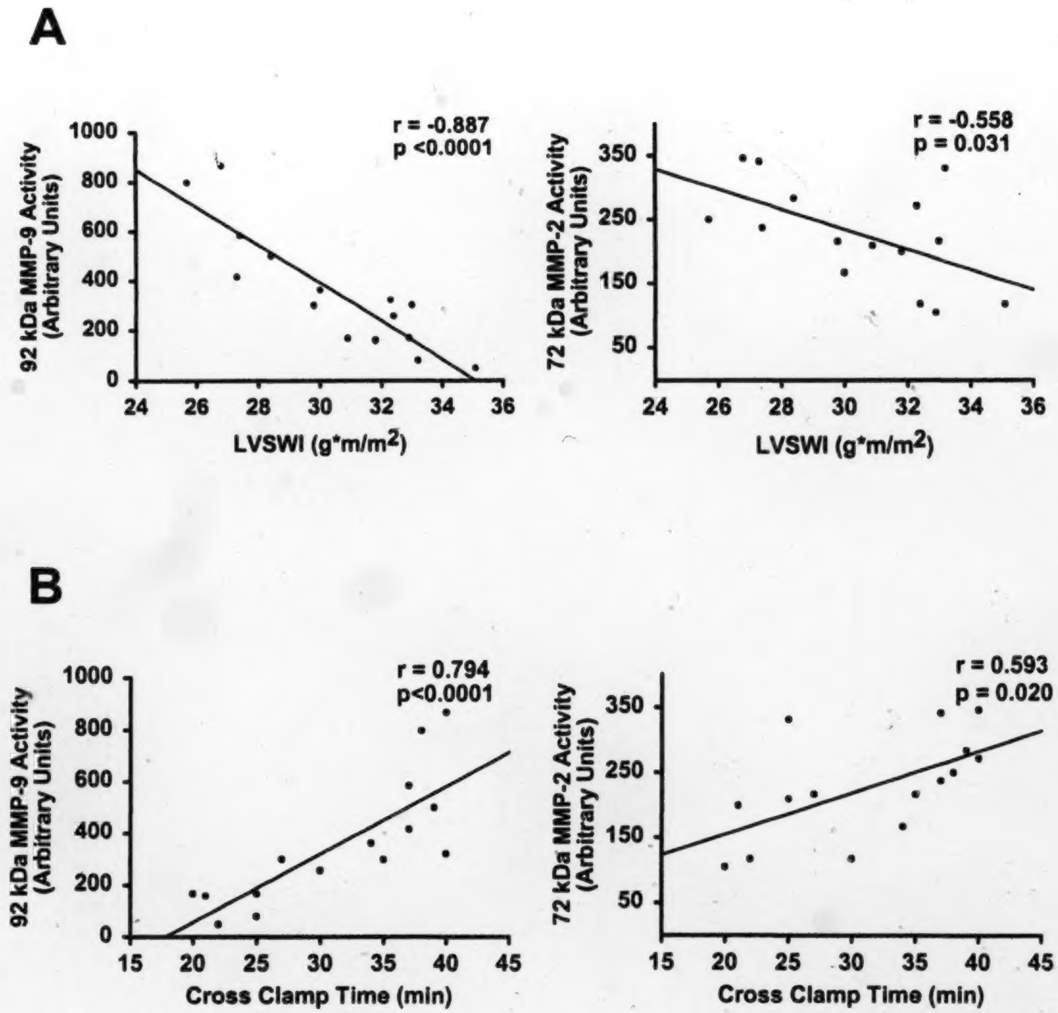

Figure 5.3 Myocardial MMP activities correlate (A) negatively with mechanical function (LVSWI) at 3 h of reperfusion and (B) positively with the duration of ischemia (cross clamp time) duration.

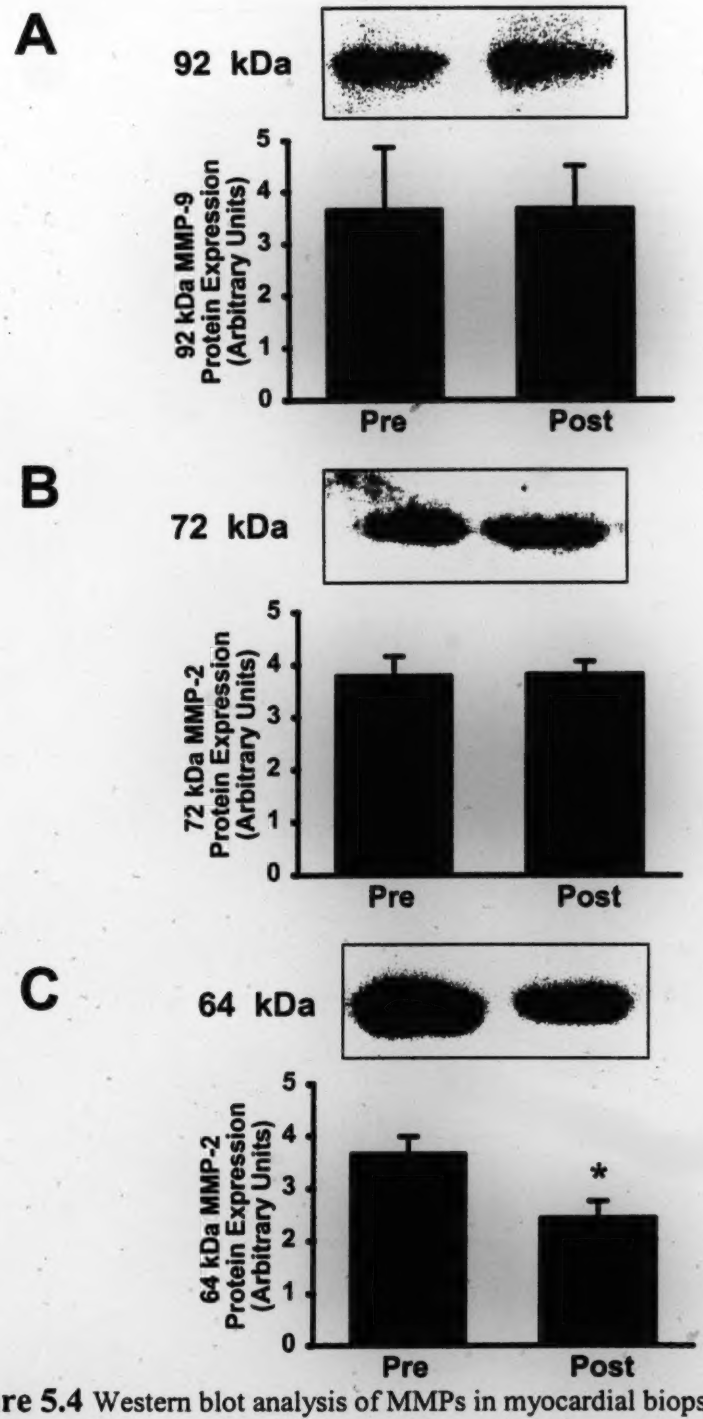

Figure 5.4 Western blot analysis of MMPs in myocardial biopsies prior to (Pre) and following ischemia-reperfusion (Post). (A-C) Representative immunoblots and quantitative results of densitometric analysis for 92 kDa MMP-9, 72 kDa MMP-2, and 64 kDa MMP-2, respectively (* $p<0.05$ vs. 'Pre' value, n=9). *(Analysis done with help of C. Schulze.)*

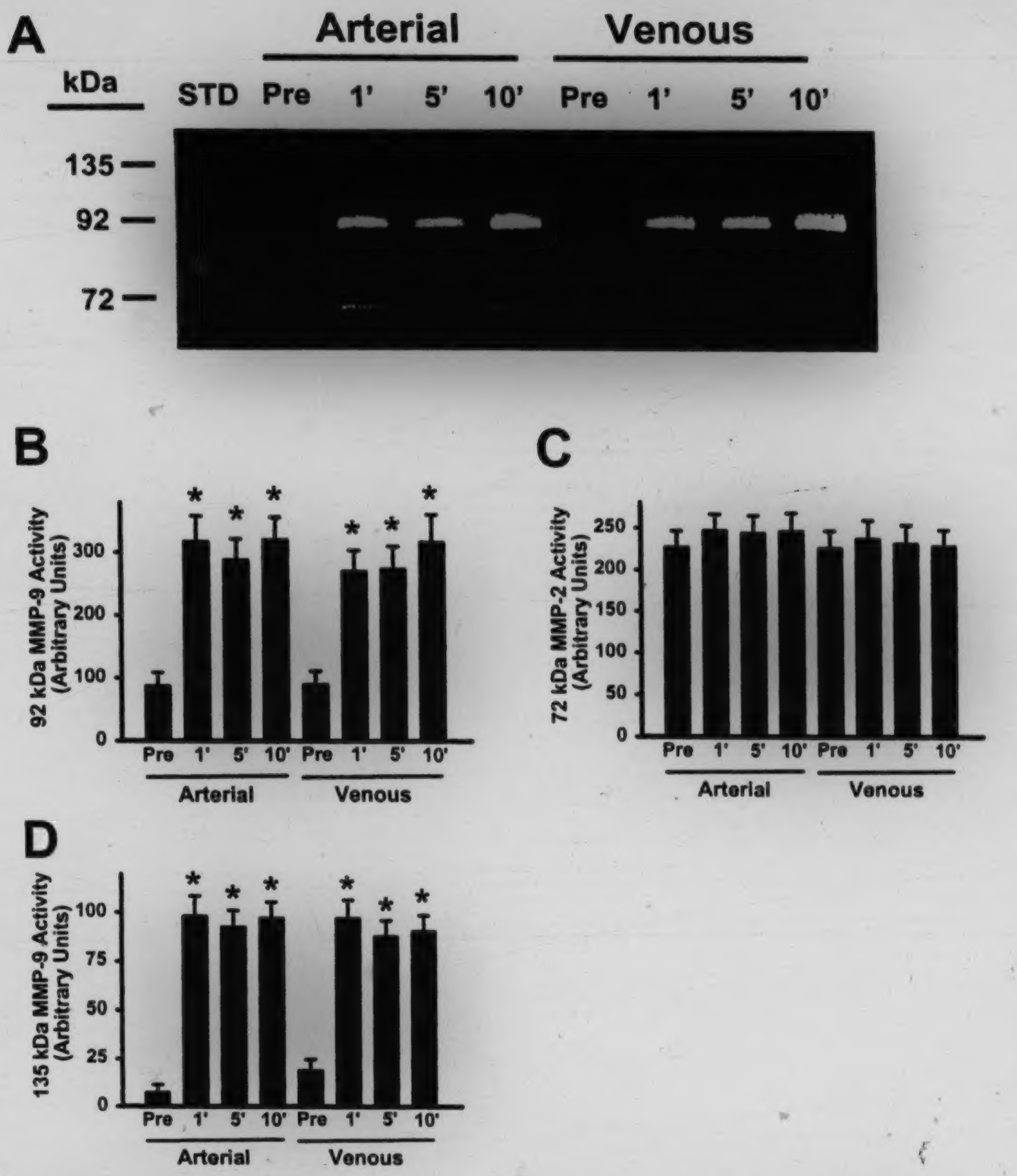

Figure 5.5 MMP activities in plasma samples prior to and following reperfusion. (A) Representative zymogram of myocardial MMP activities in one patient. HT-1080 cell culture medium was loaded into the left lane as a standard (STD). The subsequent lanes represent plasma MMP activities in radial artery (Arterial) and coronary sinus (Venous) samples. Samples were collected following initiation of CPB but prior to ischemia (Pre) and post reperfusion at 1, 5, and 10 min. 135 kDa MMP-9, 92 kDa MMP-9, and 72 kDa MMP-2 activities were detected. (B – D) Gelatinolytic activities were quantified by densitometric analysis (* $p<0.05$ vs. respective 'Pre' value, n=15). *(Zymography done with help from C. Schulze.)*

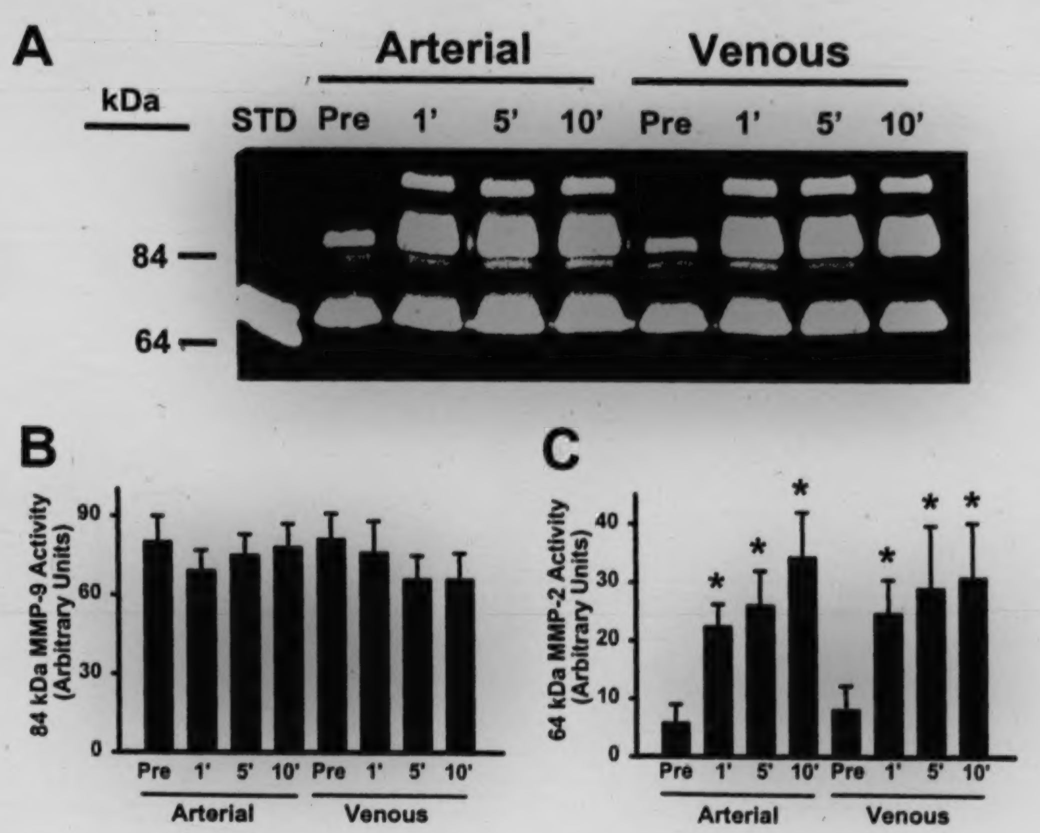

Figure 5.6 MMP activities in plasma samples prior to and following reperfusion. (A) Representative zymogram of myocardial MMP activities in one patient. Samples were incubated for a longer period in zymography buffer in order to reveal and quantify weaker bands of gelatinolytic activity as seen in Figure 5. From this zymogram, 84 kDa MMP-9 and 64 kDa MMP-2 could be detected and quantified in the plasma samples. (B - C) Densitometric analysis of 84 kDa MMP-9 and 64 kDa MMP-2 (* = $p<0.05$ vs. respective 'Pre' value, n=15). *(Zymography done with help from C. Schulze.)*

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CHAPTER 6

CONCLUSIONS, LIMITATIONS, AND FUTURE DIRECTIONS

6.1 Conclusions

I have demonstrated that cardiovascular matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) are regulated in acute inflammatory distress seen in endotoxemia and ischemia-reperfusion (I/R) injury.

In the model of sublethal endotoxemia, I found that overall cardiovascular function, assessed by mean arterial blood pressure, was significantly reduced following lipopolysaccharide (LPS) administration. This depression was most significant 6 and 12 h after LPS administration. By 24 h post LPS administration, blood pressure remained significantly depressed, however, it appeared that the rats were recovering. These observations confirmed that this was a model of reversible inflammatory cardiovascular dysfunction.

These changes in blood pressure were accompanied by changes in myocardial and plasma MMP-2 and MMP-9 activities. Contrary to our initial hypothesis, MMP-2 activity was significantly reduced in both the heart and plasma following LPS administration. Several explanations exist for our observations. First, MMP-2 may be actively secreted from the heart (as it is following I/R injury in isolated rat hearts) and this cannot be detected in the large pool of plasma MMPs. Alternatively, since endotoxemia is a state of overall increased proteolysis (due in part to the inappropriate activation of the coagulation cascade), the synthesis of secretable MMP-2 may be depressed as a protective mechanism.

The increase in circulating MMP-9 activity was expected given the evidence linking inflammation with an induction of MMP-9. However, this was the first time changes in MMP-9 were noted in any animal model used in our lab.[1,2] The importance of

MMP-9 in this model was demonstrated by the inverse correlation between this proteinase's activity and mean arterial blood pressure. Although circulating MMP-9 had been previously shown to be increased in human endotoxemia and in non-surviving septic shock patients,[3,4] this is the first correlation of circulating MMP-9 activity with acute cardiovascular dysfunction. If these observations translate clinically, MMP-9 could prove to be prognostic indicator of septic severity.

Next, I addressed the question of whether MMPs are actively secreted from the endotoxemic heart, and whether MMP inhibition is cardioprotective in this situation. I first characterized the cardiac dysfunction in this model, and found that cardiac work (i.e. cardiac output x peak systolic pressure) was significantly depressed 6 h following LPS injection. This dysfunction was associated with decreased ventricular and perfusate MMP-2 activity and increased MMP-9 activity in the perfusate as compared to control hearts. Putting this observation in the context of the previous study, the release of MMP-9 from the heart may contribute to increases in circulating MMP-9 activity. Moreover, the decrease in MMP-2 secretion from the heart may also contribute to the overall decreased levels of circulating MMP-2 at 6 h post LPS administration.

When MMP inhibitors were administered after LPS in these animals, their cardiac function was significantly improved. Although the MMP inhibitors had no effect on the loss of MMP-2 activity, the increase in perfusate MMP-9 was ameliorated. From these data, it could be speculated that MMP-2 does not have an important role in endotoxemic cardiac dysfunction. This may be the case, however, an early activation and release of MMP-2 may have been missed in our observations. Such an early activation and release is supported by a previous investigation in our lab which demonstrated that isolated rat hearts perfused with proinflammatory cytokines release increased amounts of MMP-2 within 30 min.[1] Based on my observations in the endotoxemic heart, the MMP inhibitors did not prevent such an activation and release, however, they would have certainly inhibited the proteolytic activity caused by such an event. This issue of an early activation and release of MMPs from the heart *in vivo* needs to be addressed in future studies.

In a separate study I investigated the role of MMPs in different models of vascular inflammatory stress. MMP inhibitors were found to decrease both LPS and IL-1β induced vascular contractile dysfunction. These results compliment the findings of the previous study since MMP inhibitors were again found to protect against inflammatory stress. *In vivo,* aortic gelatinolytic activities were increased 6 h after LPS administration. MMP-9 and MMP-2 activities were increased relative to their protein content, and TIMP-4 protein content was decreased while TIMP-1 protein content was increased. These results demonstrate for the first time that MMPs and TIMPs are regulated in the endotoxemic vasculature *in* vivo. The implications of these *in vivo* alterations remains to be determined, however, the ability of MMP inhibitors to protect against vascular dysfunction *ex vivo* suggests that MMP activity contributes to inflammatory vascular dysfunction.

In all three studies of the endotoxemic rat, MMP-9 activity appeared to be associated with cardiovascular dysfunction. Circulating MMP-9, cardiac release of MMP-9, and vascular MMP-9 were all increased 6 h after LPS administration. These data support an important role for MMP-9 in the endotoxemic cardiovascular dysfunction, and help explain why other groups have observed significant resistance to lethal endotoxemia in MMP-9 knockout mice.[5] The exact targets of MMP-9 within this pathology remain to be ascertained, and drugs specifically targeting MMP-9 should be developed and tested in models of endotoxemia and septic shock.

My final two studies dealt with myocardial I/R as a model of inflammatory stress. In the isolated rat heart model of ischemia-reperfusion, I demonstrated that ischemic preconditioning decreases the activation and release of MMP-2 from the myocardium. This decreased loss of MMP-2 was accompanied by a preservation of MMP-2 activity and protein content within the ventricular tissue. The significance of this study is best understood in the context of previous studies which established MMP-2 as an end effector of I/R injury in the rat heart. In light of these studies, my observations in preconditioned hearts fit in well with our overall hypothesis that MMP-2 is an end effector of I/R injury. Since ischemic preconditioning is a well-established model which reduces peroxynitrite formation and protects against I/R injury, it would be predicted that MMP-2 activation and release would be blunted with this protocol. Since MMP-2 activation was decreased, it is likely that activation of this proteinase lies downstream of the cellular processes which are dampened by ischemic preconditioning prior to I/R injury. However, many questions remain in this model, some of which are elaborated in Section 6.2.4.

My investigations of MMP activity in myocardial I/R injury were continued in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass. In this clinical situation it was found that MMP-2 and MMP-9 activities were significantly increased following reperfusion of the myocardium. In the plasma, MMP-9 and lower molecular weight MMP-2 activities were increased, but transcardiac release or uptake of this activity was not observed. The activation of myocardial MMPs in many ways mimics what was seen in our isolated perfused rat heart model of I/R, however, several key differences exist. First, MMP-9 activity, which was not detectable in isolated rat hearts subjected to I/R, was abundant in the human heart prior to I/R. Moreover, the increase in MMP-9 activity following I/R outstripped that of MMP-2. Second, there was no detectable release of MMP activity from the human myocardium following I/R injury. In the isolated rat heart model of I/R we speculated that release of MMP-2 is a protective mechanism in order to export inappropriate proteolytic activity (see Figure 1.3, Chapter 1). In light of this hypothesis, the absence of MMP release may in fact contribute to prolonged cardiac dysfunction. Alternatively, perhaps the release of MMPs is delayed in human hearts, a notion which is supported by a previous study.[6]

Despite these differences between animal and clinical models of myocardial I/R two important similarities exist. First, the magnitude of MMP activation correlated directly with the ischemic time. Second, the magnitude of MMP activation correlated inversely with the resulting cardiac dysfunction. These relationships were first clearly demonstrated in our animal model, and it was subsequently demonstrated that MMP inhibition protected against I/R induced dysfunction. These parallels lead me to speculate that MMP inhibition may be a viable strategy to prevent I/R induced cardiac dysfunction in clinical settings. As a final comment to the MMP side of this story, it should be noted that this was the first demonstration that MMP activity within the myocardium correlates with cardiac function in humans. This is particularly exciting given the number of studies which have tried to implicate MMPs in various cardiovascular pathologies.

Finally, I also studied the effects of myocardial I/R injury on the TIMPs. Myocardial TIMP-1 was decreased while TIMP-2 and TIMP-4 were not altered. Again, several important differences exist between these observations and our results in the isolated rat heart model of myocardial I/R injury. First, TIMP-1, -2, and –4 protein content were readily detectable in human hearts, whereas TIMP-4 inhibitory activity was the main TIMP found in rat hearts.[7] Second, only a loss of TIMP-1 was noted in human hearts with no changes in TIMP-2 and –4, whereas TIMP-4 was lost in rat heart following I/R. Despite these differences, the overall imbalance between MMP proteolytic activity and TIMP inhibition exists in both models.

6.2 Limitations

6.2.1 General Limitations

Although the data presented in my thesis implicates MMP activity in different aspects of inflammatory cardiovascular dysfunction, a number of limitations exist.

Throughout my thesis, gelatin zymography was used as the primary measure of MMP activity. Although this technique is well established, extremely sensitive, and highly reproducible, several limitations exist. First, gelatin zymography only detects gelatin degrading MMP-2 and MMP-9 activities. Thus, important contributions of other MMPs to inflammatory cardiovascular dysfunction may have been overlooked. Second, the underlying theory of gelatin zymography is problematic It is widely accepted that gelatin zymography is a "total activity" assay for MMP-2 and –9 since the reducing conditions of the gel activates all latent activity and is thought to dissociate any MMP-TIMP complexes. If this were the case, activity as measured by zymography would always parallel measures of protein content by immunoblot. However, my data from Chapters 2, 3, and 5 (in which zymographic activity did not always correlate with protein content) suggests that zymography may be more of a net activity assay than a total activity assay. This view of zymography remains to be confirmed.

Another shortfall found in several chapters is the lack of TIMP inhibitory activity measurement. Although both MMP activity and protein content can be measured, no reliable assay exists to measure TIMP activity in complex tissues. Thus, I can only speculate that oxidants may have decreased TIMP inhibitory activity under conditions of inflammatory stress.

The model of endotoxemia used in Chapters 2 and 3 also has several issues which need to be elaborated. Many scientists have dismissed endotoxemia as an irrelevant model of inflammatory stress. This, in part, is due to the large number of therapies that have proven to be efficacious in endotoxemia, only to go on and fail in clinical trials against septic shock.[8,9] Since endotoxemia involves administration of only the outside coat of bacteria, it is not surprising that this model does not completely mimic the complexities of clinical septic shock. However, I believe no one animal model will ever truly reproduce a clinical condition which can be caused by gram negative bacteria, gram positive bacteria, fungi, parasites, etc. and has such a varied presentation. Moreover, one should not lose sight of the fact that endotoxemia reproduces many aspects of the proinflammatory response and cardiovascular dysfunction seen in sepsis.

6.2.2 Limitations in Chapter 2

In this rat model of endotoxemia the increases in circulating MMP-9 were never fully explored. It is interesting that MMP-9 activity correlated inversely with blood pressure, but it was never demonstrated whether this increase was a cause or consequence of decreased blood pressure. As well, although it was speculated that activated neutrophils are a major source of circulating MMP-9 in endotoxemia, the source of increased MMP-9 was never confirmed.

Another major limitation in this chapter was the *ex vivo* assessment of cardiac function by means of isolated heart perfusion. This technique may have affected our results since it measures heart function outside its normal physiological environment. The heart is free of neurological and hormonal influences, and is perfused with a crystalloid that is quite different from blood.

A third limitation in this study is the selection and timing of MMP inhibitors. The MMP inhibitors (Ro 31-9790 and doxycycline) are broad-spectrum MMP inhibitors, thus, effects on MMPs other than MMP-2 and –9 may have contributed to the observed cardioprotection. As well, these inhibitors were administered half an hour after LPS administration, prior to the appearance of overt signs of endotoxemia. Translating this approach to a clinical modality will be challenging, since it is difficult to predict whether a patient will develop septic shock or not.

Finally, the specific targets of MMP activity within the endotoxemic cardiovascular system were never identified.

6.2.3 Limitations in Chapter 3

Aside from the limitations already discussed at the end of Chapter 3, several other issues should be raised. First, a more 'direct' model of LPS induced dysfunction could have been used. However, the paper which initially developed this model[10] has been cited over 500 times. Thus, it appears that this model of 'ambient LPS' is generally accepted.

Second, the model of proinflammatory cytokine stress used only IL-1β as a stimulus. As described in Chapter 1, IL-1β is only one of many cytokines which are produced in endotoxemia and septic shock. Thus, using IL-1β alone may not reflect the vascular dysfunction seen *in vivo* in sepsis and endotoxemia. An alternative to using IL-1β would have been the application of a cytokine cocktail,[1,11] but this would have masked the contribution of the individual cytokines to the dysfunction.

Third, the contribution of MMPs to *in vivo* vascular dysfunction was never explored. Whether the increases in MMP activity are a cause or consequence of endotoxemic vascular dysfunction remains to be determined.

6.2.4 Limitations in Chapter 4

The principal limitation with Chapter 4 is the purely observational nature of this study. It is difficult to tell whether MMP-2 plays a role in the preconditioning process, so future studies will need to address this issue. Another shortcoming of this study is the lack of functional data. On this point, it should be noted that the isolated heart perfusions were performed by a perfusionist experienced in the ischemic preconditioning protocol, and that functional protection has been consistently demonstrated by this perfusionist in previous studies.[12,13] Despite these limitations, this study provides another important piece of the puzzle which suggests that MMP-2 is involved in I/R injury.

6.2.5 Limitations in Chapter 5

Aside from the limitations already discussed at the end of Chapter 5, I believe a major shortcoming of this study is the failure to identify the target of increased MMP activity. Although we have demonstrated in isolated rat hearts that MMP-2 cleaves troponin I, the limited tissue available in the present clinical study prevented me from investigating the exact targets of MMP activity in human hearts.

6.3 Future Directions

Many questions remain unresolved in the different chapters of this thesis. In the animal model of endotoxemia, the 'source and sink' of MMP-9 need to be determined. In other words, the cells/organs responsible for increased circulating MMP-9 need to be identified, and the targets of this increased proteolytic activity should be investigated. As well, the contribution of MMP-9 to the overall cardiovascular dysfunction should be determined. Since MMP-9 activity is increased in the heart and vasculature during endotoxemia, I have suggested that this MMP plays an important role in this dysfunction. This hypothesis could be easily addressed by determining whether MMP-9 knockout mice are protected against endotoxemic or septic cardiovascular dysfunction.

Since the applicability of the endotoxemia model will always be questioned, I believe some of the experiments outlined in this thesis should be repeated in a more widely accepted model, such a cecal ligation and puncture. This model is regarded as a 'true' animal model of sepsis, and thus changes in MMP activity in this model might be more applicable to sepsis in humans.

In ischemic preconditioning, the exact role MMP-2 plays in this process remains to be determined. Since peroxynitrite is a preconditioning stimulus, it follows that MMP-2 may also be a preconditioning stimulus. This possibility could be tested by intermittently infusing MMP-2 for short periods of time into an isolated perfused rat heart prior to a test I/R injury.

In the human study of I/R injury many exciting questions have arisen. First, like the studies in previous chapters, the exact target of MMP activity remains to be determined. Troponin I degradation has been proposed as a possible mechanism for I/R injury in humans, and the proteolysis of troponin I has been demonstrated in patients subjected to coronary artery bypass grafting with cardiopulmonary bypass.[14,15] The increase in MMP activity found in the human heart following I/R suggests that MMPs may cause this degradation of troponin I.

Second, in this study myocardial collagenase activity was also increased. This is the first time collagenase activity has been demonstrated to be acutely increased following I/R injury, however, the MMP species responsible for this increase need to be identified.

Finally, general inhibition of MMPs should be explored as a prophylactic therapy against I/R cardiac dysfunction seen after cardiac surgery. Since doxycycline is a safe and inexpensive broad spectrum MMP inhibitor, it could be easily and safely added to cardioplegic solution. It will be interesting to see whether MMP inhibition translates into a viable clinical modality to treat acute inflammatory cardiovascular dysfunction.

6.4 References

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